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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 31/18, 31/185, 31/255
A61K 31/795, 31/80, 31/765
A61K 31/19, 31/66
C07C 309/24, 143/00, 311/13
C07F 9/38, 9/40, C07C 65/34

A1

(11) International Publication Number: WO 94/03164

(43) International Publication Date: 17 February 1994 (17.02.94)

(21) International Application Number: PCT/US93/07366

(22) International Filing Date: 5 August 1993 (05.08.93)

(30) Priority data:
928,108 6 August 1992 (06.08.92) US
072,566 4 June 1993 (04.06.93) US

(60) Parent Applications or Grants
(63) Related by Continuation
US 928,108 (CIP)
Filed on 6 August 1992 (06.08.92)
US 072,566 (CIP)
Filed on 4 June 1993 (04.06.93)

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(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: INHIBITION AND TREATMENT OF INFECTION BY ENVELOPED VIRUS WITH CALIX(N) ARENE COMPOUNDS

(57) Abstract

A method for inhibiting cell infection by an enveloped virus, by administering to an infection site, a therapeutically effective amount of a calix(n) arene compound derivatized, at its ring positions meta to the bridge attachments to the ring, with polar substituent having a terminal carboxylate, phosphate, or sulfonate groups, including esters and amides which are cleavable *in vivo*. The compound may be administered orally, or topically, e.g., for treatment of herpes virus. The invention also includes a method of inhibiting infection by sexually transmitted enveloped viruses, by topically administering to an area of likely sexual contact a composition containing a prophylactically effective amount of a macrocyclic compound such as described above.

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INHIBITION AND TREATMENT OF INFECTION BY ENVELOPED VIRUS WITH CALIX(N)ARENE COMPOUNDS

1. Field of the Invention

The present invention relates to a method for inhibiting cell infection by enveloped viruses, and more particularly, to a method which employs a defined class of calix(n)arene compounds; and in a related aspect, to a method for inhibiting infection by sexually transmitted enveloped viruses.

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3. Background of the Invention

The challenge in developing an effective therapy and prophylaxis for viral disease is to achieve inhibition of viral processes without producing extreme side effects and preferably without inducing viral resistance. Since viral replication requires use of the cellular apparatus of the host, treating virus infection by inhibiting viral replication can be lethal to the infected host cells as well. Ideally, the virus should be destroyed or inactivated in the host prior to its invasion of host cells. This is normally accomplished, with varying degrees of success, by the host's immune system, but this mechanism requires an earlier immune response, either by a prior infection or by vaccination. Further,

many viruses, such as Herpes Simplex viruses (HSV) are able to effectively elude a host's immune systems, and at least one virus, the human immunodeficiency virus (HIV) is known to cripple the host's immune system (Gottlieb).

Currently, the most widely used anti-viral agents are nucleoside analogs. This class of drugs acts by disrupting viral replication, either by inhibiting enzymes required for nucleic acid processing, or by producing defective viral genomes, such as by premature termination of replication. As an example, acyclovir, a purine analog used in treating a variety of viral diseases, including herpes simplex virus-1 (HSV-1) and herpes simplex virus-2 (HSV-2) inhibits viral replication at several key points, including inhibition of viral thymidine kinase and DNA polymerase, and DNA strand elongation (Elion). Ribavirin, another purine analog, is the drug of choice in treating respiratory syncytial viruses (RSV) infection. This compound appears to act by reducing cellular GTP levels, blocking the action of several GTP-dependent viral processes (Smith). To date, the most common drug treatment of HIV infection is with zidovudine (Azidothymidine; AZT), a thymidine analog which is particularly effective against human retroviruses. AZT acts with high affinity to block viral RNA-dependent DNA polymerase (reverse transcriptase), but does also block human DNA- polymerase and causes chain termination (Mitsuya).

Other nucleic acid analogs include ganciclovir, vidarabine, idoxuridine, trifluridine and foscarnet (an inorganic phosphate analog). As indicated above, all of these drugs, by blocking viral replication, also have the capacity to disrupt normal host

replication and/or DNA transcription processes as well (see, e.g., Martin).

Understanding of the mechanisms of infection and replication of viruses has lead to alternate drug therapies, including attempts to block viral entry into cells, alter protein synthesis at the host ribosomes, complexation of viral DNA/RNA, and immunomodulation. Interferons are glycoproteins which have complex actions including enhancement of certain immune responses as well as direct antiviral action. They are more competent in preventing infection, rather than treating established viral infection, and their use leads to undesirable problems including acute, serious discomfort, bone marrow suppression, viral resistance, and development of host immune response to the interferon.

Treatment with "anti-sense" polymers of nucleic acids is a method in which the particular viral genome is the select target. The treatment provides a highly discriminating approach which would be expected to have minimal side-effects; its use as a therapeutic is hampered by problems of targeting, introduction into cells, and the quantity of material that would be required to block each strand produced. Agents which bind to and interfere with host ribosomal protein synthesis will block viral replication. These include the toxin ricin, various plant proteins such as pokeweed anti-viral protein, alpha sarcin, and other low molecular weight compounds. The weakness with the use of these materials is their lack of selectivity. In the treatment of HIV, additional therapy has been developed by specifically targeting the unique retroviral enzyme, reverse transcriptase. Non-retroviral systems do not produce or

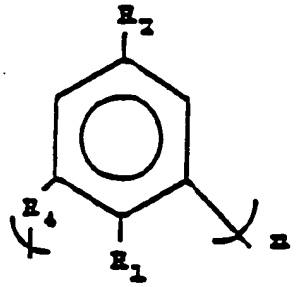
use this enzyme, but the virus cannot replicate without it.

In some instances, an understanding of structural aspects of the mechanisms of replication of viruses has provided additional drug therapies. Certain viruses, including orthomyxoviruses and paramyxovirus, herpes viruses, togaviruses and retroviruses, contain a viral envelope which surrounds the viral capsid and nucleic acid. During cell infection by an enveloped virus, the plasma membrane of the host cell is altered to include some viral-coded proteins and, as the viral nucleoprotein core exits the host cell in which it was assembled, it becomes enveloped with the modified membrane, thus forming the viral envelope. Because this structure is unique to host cells when they are virally infectious and distinct from normal cells, it can serve as an additional target for therapeutic assault.

4. Summary of the Invention

The present invention includes a method for treating infection by an enveloped virus. The method includes administering to the site of infection a therapeutically effective dose of a calix(n)arene compound which is derivatized, at its ring positions meta to the bridge attachments to the ring, with polar substituents having terminal carboxylate, phosphate, sulfinate, or sulfonate groups.

In one general embodiment, the calix(n)arene has the general structure:



where n = 4-10, R_1 is OH, =O, an alkyl or aryl ether, an ester, an acid, or a mixture thereof; R_2 is a polar substitutent with a terminal carboxylate, phosphonate, sulfinate, or sulfonate group, including cleavable esters and amides thereof; and R_4 is >CHR", ≥ CR", or a mixture thereof, where R" is H or a carboxylate group. R_1 is preferably OH, or, in a partially oxidized form of the compound, a combination of OH and =O.

In one specific embodiment, the compound has the structure shown above, where n = 4, 6, or 8; R_1 is OH or =O or a mixture thereof; R_2 is as above; and R_4 is $>CH_2$ or ≥CH, or a mixture thereof.

In a more specific embodiment, R_2 has the form: $(CH_2)_mR_2'$, where m= 1-3, and R_2' is a sulfonate group of the form SO_3R or SO_2NRR', where R and R' are each H or a lower alkyl group.

In another embodiment, R_2 has the form: $(CH_2)_m$-R_2', where m= 1-3, and R_2' is a sulfinate group of the form SO_2R or S(=O)NRR', where R and R' are each H or a lower alkyl group.

In another embodiment, R_2 has the form: $(CH_2)_m$-R_2', where m= 0-3, and R_2' is a carboxylate group of the form CO_2R or C(O)NRR', where R and R' are each H or a lower alkyl group.

In another embodiment, R_2 has the form:

$(CH_2)_m$-R_2', where m= 0-3, and R_2' is a phosphonate group of the form $PO(OR)_2$, PO(OH)(OR), PO(OR)(NRR'), or $PO(NRR')_2$, where R and R' are each H or a lower alkyl group.

In another aspect, the invention contemplates novel calix(n)arene compounds of the type described above, and having sulfinate, sulfonate, phosphonate, and carboxylate terminal groups.

In one embodiment, the novel compounds include oxidized and partially oxidized calix(n)arenes having the general structure shown above, where R_1 is OH, =O, an alkyl or aryl ether, an ester, an acid, or a mixture thereof, and at least one of the R_1 groups in the calix(n)arene compound is =O; R_2 is a polar substituent with a terminal carboxylate, phosphonate, sulfinate or sulfonate group; and R_4 is $>CH_2$ or $\geq CH$, or a mixture thereof. In a preferred embodiment, R_1 is OH or =O, and at least one of the R_1 groups in the calix(n)arene compound is =O.

In another embodiment, the novel compounds include calix(n)arenes having the general structure shown above, where n = 4-10; R_1 is OH, =O, an alkyl or aryl ether, an ester, an acid, or a mixture thereof; R_2 is a polar substitutent with a terminal carboxylate or sulfinate group, and R_4 is $>CH_2$ or $\geq CH$, or a mixture thereof.

The compound may be administered orally, for treatment, for example of human immunodeficiency virus (HIV) respiratory syncytial virus (RSV), or herpes simplex viruses HSV-1 or HSV-2.

The compound may be administered by inhalation, for treatment of respiratory syncytial virus, and topically for treatment of HSV-1 or HSV-2. Other

modes of administration, e.g., intravenous, are also contemplated.

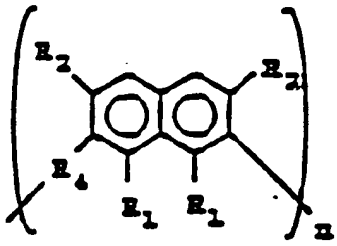
Also contemplated is a method which employs a combination of a calix(n)arene compound and an antiviral nucleoside analog compound for treating viral infection. The calix(n)arene and nucleoside analog compounds may be formulated in an ointment vehicle, for topical administration, e.g., in treating lesions due to HSV-1 or HSV-2. Alternatively, the compounds may be formulated in liquid or tablet form for oral administration, for treatment of systemic viral infection, or in solution form for systemic administration.

In another aspect, the invention includes a method of inhibiting infection by a sexually-transmitted enveloped virus. In the method, a prophylactically effective amount of a macrocyclic compound is administered topically to an area of likely sexual contact. The macrocyclic compound is composed of aryl ring subunits which are connected by ring-attached bridge linkages which form a continuous chain of connected atoms making up the backbone of the macrocycle, and which contain negatively charged substituents on non-backbone atoms of the aryl subunits. Preferably, the negatively charged substituents include a sulfonate group, a sulfinate group, a carboxylate group, or a phosphonate group. In one embodiment, the compound includes amide or ester forms of the negatively charged substituent, which can be cleaved to a negatively charged form *in vivo*.

The ring subunits in the macrocyclic compound preferably include naphthalene subunits having 3- and 6-position sulfinate or sulfonate groups; and/or phenyl subunits having 4-position negatively charged

substituents, where the bridge linkages are between the 2 ring-carbon position of one naphthalene or phenyl group, and the 7 ring-carbon group of an adjacent naphthalene group or 5 ring-carbon position of an adjacent phenyl group.

In one general embodiment, the macrocyclic compound includes at least four naphthalene subunits, each having polar groups at 1- and 8-position, sulfinate or sulfonate groups at 3- and 6-positions, and bridge linkages between the 2 ring-carbon position of one subunit and the 7 ring-carbon position of an adjacent subunit. One preferred compound of this type has the general structure:

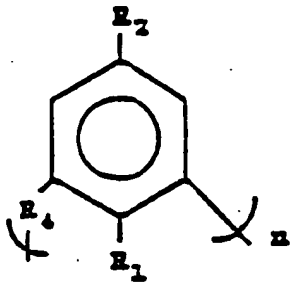


where n = 4, 6, or 8, R_1 is OH, =O, an alkyl or aryl ether, an ester, an acid, or a mixture thereof, R_2 is a sulfonate group or sulfinate group, and R_4 is >CHR", ≥CR", or a mixture thereof, where R" is H or a carboxylate group.

In a more specific embodiment, R_1 is OH, =O or a mixture thereof, R_2 is a sulfonate group, and R_4 is $>CH_2$ or ≥CH, or a mixture thereof.

In another general embodiment, the macrocyclic compound includes at least four phenyl subunits having negatively charged substituents at 4-positions, and bridge linkages between a 2 ring-carbon position of one phenyl subunit and a 5 ring-carbon position of an adjacent phenyl subunit. One

preferred compound of this type has the general structure:



where n, R_1, R_2, and R_4 are as above.

In a more specific embodiment, R_2 has the form: $(CH_2)_mR_2'$, where m= 0-3, and R_2' is a sulfonate group.

In another specific embodiment, R_2 has the form: $(CH_2)_mR_2'$, where m= 0-3, and R_2' is a sulfinate group.

In another specific embodiment, R_2 has the form: $(CH_2)_mR_2'$, where m= 0-3, and R_2' is a carboxylate group.

In yet another specific embodiment, R_2 has the form: $(CH_2)_mR_2'$, where m= 0-3, and R_2' is a phosphonate group.

Also contemplated is a method which employs a composition containing a macrocyclic compound such as described above and an antiviral nucleoside analog compound.

Compositions in accordance with the invention are administered topically for inhibiting transmission of viral infection between sexual partners. The compositions may take the form of a lubricating jelly, suppository, for example, and include a vehicle in which the macrocyclic compound is carried. In addition, the compositions may be applied to the surfaces of physical barrier-type devices as a prophylactic measure against infection.

In another aspect, the invention includes a lubricating jelly composition for use in inhibiting infection by sexually transmitted enveloped viruses. The jelly composition includes a lubricating jelly vehicle, and dissolved in the vehicle, a macrocyclic compound such as described above.

In another aspect, the invention includes a physical-barrier type device in combination with a macrocylic compound of the type described above, for use in inhibiting infection by sexually transmitted enveloped viruses. In one embodiment, the device is coated with a lubricating jelly composition containing a macrocyclic compound such as described above.

Sexually transmitted enveloped viruses against which the compounds of the invention are prophylactically effective include hepatitis delta virus (HDV), hepatitis B virus (HBV), hepatitis C virus (HCV), papillomavirus, herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), HIV-1, HIV-2, HTLV-I and HTLV-II.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 shows the general structure of a macrocyclic compound composed of naphthalene subunits, for use in the present invention;

Figures 2A and 2B show non-oxidized (2A) and partially oxidized (2B) forms of the Figure-1 structure, where n=4 and the subunit is chromotropic acid;

Figures 3A and 3B illustrate two general methods of synthesis of a macrocyclic compound like the one shown in Figure 2A;

Figures 4A and 4B show an unoxidized (4A) and partially oxidized (4B) macrocycle with mixed phenyl and sulfonated naphthalene subunits;

Figure 5 illustrates reaction methods for converting the sulfonic acid substituents of macrocyclic chromotropic acid to glycyl sulfonamide and sulfonamide groups;

Figure 6 illustrates a reaction method for converting sulfonate groups in a macrocycle containing chromotropic acid subunits to a sulfinate salt or sulfinate methyl or aryl ester;

Figure 7 shows the general structure of a macrocyclic compound composed of phenyl groups, for use in the present invention;

Figure 8 shows a non-oxidized form of the Figure-7 structure, where n=4 and the subunit is para-sulfonic acid phenol;

Figures 9A and 9B illustrate general methods of synthesis of non-oxidized and partially oxidized forms of the Figure 8 compound;

Figure 10 shows a reaction scheme for replacing the ring hydroxyl groups in the Figure-8 compound with acetyl groups;

Figure 11 shows a reaction for converting sulfonic acid substituents to glycyl sulfonamide groups in a phenyl-subunit macrocyclic compound;

Figure 12 shows a reaction scheme for producing a macrocylic compound like that shown in Figure 8 but with carboxylic acid-containing bridge linkages;

Figure 13 shows a reaction scheme for replacing hydroxyl groups in the Figure-8 compound with carboxylic acid groups.

Figure 14 shows a reaction scheme for producing a calix(n)arene from a para-tert-butyl-precursor;

Figure 15 shows a reaction scheme for preparing a calix(n)arene having para-carboxyl substituents;

Figure 16 shows a reaction scheme for preparing a calix(n)arene having carboxyl substituents linked to the para position by a methylene linker;

Figure 17 shows a reaction scheme for preparing a calix(n)arene like that of Figure 16 but where the carboxyl substituents are linked to the para position by an ethylene linker;

Figure 18 shows a reaction scheme for preparing a calix(n)arene having para-phosphonate substituents;

Figure 19 shows a reaction scheme for preparing a calix(n)arene having a phosphonate substituent linked to the para position (C-4) by a methylene group;

Figure 20 shows a reaction scheme for preparing a p-2-bromoethyl-O-tosyl-calix(n)arene as a precursor for preparing other calix(n)arene derivatives;

Figure 21 shows a reaction scheme employing the bromoethyl-calix(n)arene of Figure 20 to prepare a calix(n)arene having a phosphonate group linked to C-4 by an ethylene group;

Figure 22 shows a reaction scheme for preparing a calix(n)arene derivative having a sulfonate group linked to C-4 by a methylene group;

Figure 23 shows a reaction scheme for preparing a calix(n)arene derivative having a sulfonate group linked to C-4 by an ethylene group;

Figure 24 shows a reaction scheme for preparing a calix(n)arene having a chlorine atom at the methylene bridge (R_4 in Fig. 7) for introduction of other substituents at the methylene bridge;

Figure 25 shows a reaction scheme for preparing calix(n)arenes like that of Figure 12, starting from the cyclized precursor from Figure 24;

Figure 26 shows a reaction scheme for preparing a calix(n)arene having a carboxymethyl group attached to the bridge methylene (structure LVIII), and a general approach for preparing a variety of calix(n)arenes having selected R groups at the methylene bridge (structure LX), using organocuprate reagents;

Figure 27 shows reaction schemes for preparing a number of calix(n)arenes having 3-sulfonylpropyloxy groups attached to calix(n)arenes at various ring positions;

Figure 28 shows a reaction scheme for preparing a macrocyclic compound having alternating phenyl and naphthyl rings;

Figures 29A and 29B are plots of HSV viral yields, as a function of drug dose, for the macrocyclic compounds KY-1 (29A) and KY-42 (29B);

Figure 30 shows the inhibition of ^{3}H-labeled HSV-1 binding to cells by the compound KY-1;

Figure 31 is a plot of the inhibition in plaque formation of HSV-1 virus when the virus is exposed to the compound KY-1 before (open squares), (ii) after (closed squares), and during (closed circles) incubation with Vero cells;

Figure 32A shows SDS-PAGE autoradiograms of HSV-1 proteins in the presence (lane A) and absence (lane B) of mercaptoethanol, and of HSV-2 proteins in the presence (lane C) and absence (lane D) of mercaptoethanol, all with bound radiolabeled KY-1, and stained marker proteins (lane E);

Figure 32B shows SDS-PAGE autoradiograms of radiolabeled KY-1 compound bound to HSV-1 glycoproteins gD (lanes A and B), gB (lanes C and D), and gC (lanes E and F);

Figures 33 (A-D) show plots of effects of topical Y-1 on epithelial damage (33A), conjunctivitis (33B), iritis (33C), and stromal disease (33D) subsequent to ocular application of HSV-1 in rabbits; and

Figures 34A and 34B show the drop in HSV-1 (34A) and HSV-2 (34B) viral yields when infected cells are exposed to increasing concentrations of Y-1 alone (solid circles), increasing concentrations of acyclovir alone (open circles), increasing concentrations of acyclovir plus 25 μg/ml Y-1 (solid squares), and increasing concentrations of acyclovir plus 50 μg/ml Y-1 (solid ovals).

Detailed Description of the Invention

I. Definitions

The terms defined in this section have the following meanings unless otherwise indicated.

An "enveloped virus" means a virus containing a proteinaceous viral envelope which surrounds the viral capsid. Such enveloped viruses include orthomyxoviruses and paramyxovirus, herpes viruses, togaviruses and retroviruses. During cell infection by an enveloped virus, the plasma membrane of the host cell is altered to include some viral-coded proteins and, as the viral nucleoprotein core exits the host cell in which it was assembled, it becomes enveloped with the modified membrane, thus forming the viral envelop.

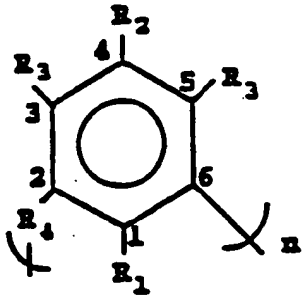
A "sexually transmitted enveloped virus" is an enveloped virus which is known or suspected to be

transmitted by sexual contact. Specific examples include hepatitis delta virus (HDV), hepatitis B virus (HBV), hepatitis C virus (HCV), papillomavirus, herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), HIV-1 (also known as HTLV-III), HIV-2, HTLV-I and HTLV-II.

An "aryl ring" subunit is a single ring or fused ring structure containing at least one aromatic ring, i.e., a 5- or 6-member ring with 6 pi electrons necessary for aromaticity. Examples include benzene, naphthalene, fused ring structures, such as tetralin, and heterocyclic structures, including fused-ring structures, such as quinoline, isoquinoline, and indole.

A "macrocyclic compound composed of aryl ring subunits" is a cyclic compound formed by linking ring atoms in aryl ring subunits to form a cyclic chain.

A "calix(n)arene" or "calixarene compound" is a macrocyclic compound having a skeletal structure of the form:



where n is preferably 4-10, and in a preferred embodiment, is 4, 6 or 8.

A partially oxidized calix(n)arene refers to a compound having the structure shown above, where at least one of the R_1 groups is =O. In a fully oxidized calix(n)arene, all of the R_1 groups are =O. An exemplary structure of a partially oxidized calix(n)arene is shown in Figure 9B. In addition,

for each R_1 group that is =O, it is to be understood that the R_4 group attached to the same aryl ring has the form ≥CR; i.e., the R_4 group is double-bonded to the aryl ring, as illustrated in Fig. 9B.

The "positions of bridge attachments to the ring" in a calixarene compound refer to ring positions 2 and 6 in each ring of the compound.

The "non-bridge positions" in a calixarene compound refer to ring positions 1, 3, 4, and 5 in each ring of the compound.

The "ring position meta to the bridge attachments" in a calixarene compound refer to ring position 4 in each ring of the compound.

A "polar substituent" refers to a radical R whose octanol/water partition coefficient is less than 1.

A "polar substituent having a terminal carboxylate, phosphonate, sulfonate, or sulfinate refers to R having the form $-CO_2^-$ or $R'-CO_2^-$ (carboxylate), $-PO_3^-$ or $R'-PO_3^-$ (phosphonate), $-SO_3^-$ or $R'-SO_3^-$ (sulfonate), $-SO_2^-$ or $R'-SO_2^-$ (sulfinate), where R' is a linear chain 1-4 atoms in length which is effective to link the associated carboxylate, phosphonate, or sulfonate group to the phenyl ring of calixarene. One preferred R' linear chain is $(CH_2)_m$, where m = 1-3.

A "carboxylate" group includes the carboxylic acid group $-CO_2^-$, carboxylate salts, and carboxylic acid esters and amides which are cleavable *in vivo*. A carboxylic acid ester has the general form $-CO_2-R$, where R is an unsubstituted lower alkyl or a substituted alkyl group, and a carboxylic acid amide has the general form CONRR', where NRR' is a secondary or tertiary amine, i.e., R and R' are each

H or lower substituted or unsubstituted lower alkyl groups. A carboxylic acid ester or amide is cleavable *in vivo* if it is hydrolysed by serum esterases or amidases, respectively, to the corresponding carboxylic group.

A "phosphonate" group includes the phosphonic acid group $-PO_3^{-2}$, including phosphonate salts, and phosphonic acid esters and amides which are cleavable *in vivo*. A phosphonic acid ester has the general form $-PO_3RR'$ where R and R′ are lower alkyl groups, or substituted lower alkyl groups, and a phosphonic acid amide has the general form $PO(OR)(NRR')$, $PO(NRR')_2$, where R and R′ are each H or a lower alkyl group. A phosphonic acid ester or amide is cleavable *in vivo* if it is hydrolysed by serum phosphatases or phosphoamidases, respectively, to the corresponding sulfonic acid group.

A "sulfonate" group includes the sulfonic acid group $-SO_3^-$, including sulfonate salts, and sulfonic acid esters and amides which are cleavable *in vivo*. A sulfonic acid ester has the general form $-SO_3R$, where R is an unsubstituted lower alkyl or substituted lower alkyl group, and a sulfonic acid amide has the general form SO_2NRR', where R and R′ are each H or a lower alkyl group. A sulfonic acid ester or amide is cleavable *in vivo* if it is hydrolysed by serum esterases or sulfoamidases, respectively, to the corresponding sulfonic acid group.

A "sulfinate" group includes the sulfinic acid group $-SO_3^-$, including sulfinate salts, and sulfinic acid esters and amides which are cleavable *in vivo*. A sulfinic acid ester has the general form $-SO_2R$, where R is an unsubstituted lower alkyl or

substituted lower alkyl group, and a sulfinic acid amide has the general form SONRR', where R and R' are each H or a lower alkyl group. A sulfinic acid ester or amide is cleavable *in vivo* if it is hydrolysed by serum esterases or sulfoamidases, respectively, to the corresponding sulfinic acid group.

The terms "sulfonate", "sulfinate", "phosphonate", and "carboxylate" include the corresponding acid forms and any pharmaceutically acceptable salts thereof.

A "negatively charged substituent" refers to a substituent which is negatively charged under physiological conditions. Exemplary groups which can impart negative charge include sulfonate groups ($-SO_3^-$), sulfinate groups ($-SO_2^-$), phosphonate groups ($-PO_3^{2-}$), and carboxylate groups ($-CO_2^-$). Preferably, the negatively charged substituent has the form $-X-SO_3^-$, $-X-SO_2^-$, $-X-PO_3^-$, or $-X-CO_2^-$, where X is a linear chain 1-4 atoms in length which is effective to link the associated carboxylate, phosphonate, sulfinate, or sulfonate group to the phenyl ring of a calixarene. One preferred chain is $(CH_2)_m$, where m = 0-3.

A "lower alkyl group" is a linear or branched alkyl group containing 1-5 carbon atoms.

A "substituted lower alkyl group" is a lower alkyl group having one or more substitutions at its carbon atoms.

II. Preparing Aryl-Subunit Macrocyclic Compounds

This section describes the synthesis of two general types of aryl macrocyclic compounds which are useful in the method of the invention. Methods for synthesizing the first type of compounds (naphthylene subunits) are discussed in section A below, and

methods for preparing compounds in accordance with the second type (phenyl subunits) are described in sections B and C. From the synthetic routes given in sections A-C, it will be apparent how macrocycles composed of mixed subunits, e.g., both naphthalene and phenyl subunits can be prepared. The synthetic methods are also generally applicable to macrocycles composed of heterocyclic subunits, particularly those having sulfonate or sulfinate substituents.

A. Macrocyclic Compounds with Substituted Naphthalene Subunits

Figure 1 shows the general structural formula of a macrocyclic compound composed of substituted naphthalene subunits, for use in the present invention. One exemplary compound of this type is shown in non-oxidized (I) and partially oxidized (II) form in Figures 2A and 2B, respectively. The compound is a tetramer of chromotropic acid (1,8-dihydroxy-3,6-disulfonic acid naphthalene) subunits linked by methylene or methine ($>CH_2$ or $\geq CH$) bridges (R_4). As seen, the methylene/methine bridges and the "interior" ring atoms (ring positions 1, 2, 7, and 8) form a continuous chain having R_1 = OH or =O polar groups attached at the 1 and 8 positions. The non-chain atoms (ring positions 3-6 on each substituent) have R_2 = sulfonate or sulfinate substituents on the 3 and 6 ring atoms. The nature of the partially oxidized structure was deduced from H^1 and C^{13} NMR studies, and from mass spectroscopy evidence.

For the discussions below (sections A-C), and for illustrating synthetic routes, usually only the non-oxidized subunit form of the compound is given. It will be understood that the compound may be partially or fully oxidized, after exposure to air

under heat and acidic conditions, i.e., contain one or more R_1 ketone (=O) groups, and a double bond between the ring and the associated bridge group (R_4), as indicated in Figure 2B. It will also be understood that the same reaction mechanisms will apply generally to the partially oxidized form of the compound, i.e., the structure shown in Figure 2B, or similar structures containing additional R_1 =O groups, except that R_1 modification reactions will typically selectively modify an R_1 -OH group, and leave the corresponding R_1 =O group intact.

As will be seen below, the compound preferably includes the chromotropic acid derivatives in which R_1 is a polar substituent, such as OH, =O, CO_2H or an ether, thioether, ester, or thioester-linked alkyl or aryl group, and combinations of these group, e.g., where the OH groups in the partially oxidized structure are substituted by one of the above groups.

R_2, as noted, is a sulfonate or sulfinate group.

R_3 is H or an uncharged or negatively charged substituent, subject to the activity constraints discussed below. Preferably, R_3 is H.

Also as will be seen below, the R_4 bridge linking the chromotropic acid derivative subunits is preferably of the form >CHR or ≥CR (indicating unsaturated bridges in the partially oxidized form), where R is H or a small carbon-containing group, such as lower alkyl, alkenyl, ketone, or carboxylic acid group, or aryl group. The bridge may also be of the form $-CH_2NR'CH_2-$, where R' is similarly H or a small carbon-containing group, such as a lower alkyl group.

Alternatively, the bridges in the macrocycle may be ring structures, including aryl ring structures, such as in the dimeric macrocycle shown in Figure 4,

or analogous structures formed by bridging through heterocyclic rings, such as pyran or furan rings.

The number of subunits may vary from 4 (e.g., Fig. 2A structure) to 8, with macrocycles containing 4, 6 and 8 subunits being preferred. In the reaction schemes described below, the macrocycle formed may include mixtures of compounds with different subunit numbers (n) values, e.g., a dominant n=4 structure (4 subunits) with additional structures containing 6 and 8 subunits.

Representative macrocyclic compounds which have been synthesized and tested for anti-viral activity are identified by their R_1, R_2, R_3, and R_4 substituents in Table 1 below. The KY and Y number in the lefthand column in the table refers to the analog designation of the corresponding compound. For example, the compound in which R_1 is OH, R_2 is SO_2NH_2, R_3 is H, and R_4 is $-CH_2-$ is designated KY-3. Although not shown in the table, the compounds may exist in a partially oxidized state in which one of more R_1 groups are =O, and adjacent bridges contain a double-bond carbon linkage to the ring.

Table 1

KY	R_1	R_2	R_3	R_4
KY-1	OH	SO_3Na	H	$>CH_2$
KY-3	OH	SO_2NH_2	H	$>CH_2$
KY-42	OH	SO_3Na	H	$>CHCO_2H$
KY-48	OH	SO_3Na	H	$>CHCHOHCH_2OH$
KY-85	OH	SO_3Na	OH	$>CHC_6H_6$
KY-97	OH	SO_3Na	H	$>CH_2CH=CH_2$
KY-110	OH	SO_3Na	H	$>CHC(O)CH_3$
KY-121	OH	$SO_2C_6H_3(OH)_2$	H	$>CH_2$
KY-123	OH	SO_2Na	H	$>CH_2$
KY-143	OH	SO_3Na	OH	$>CH_2$
KY-147	OH	SO_2NHCH_3	H	$>CH_2$
KY-148	OH	SO_2NHEt	H	$>CH_2$
KY-151	OCH_3	SO_3Na	H	$>CH_2$
KY-158	OH	SO_2CH_3	H	$>CH_2$
KY-171	OH	SH	H	$>CH_2$
KY-175	OH	SO_3CH_3	H	$>CH_2$
KY-176	OH	$SO_2NHC_6H_6$	H	$>CH_2$

Table 1

KY	R_1	R_2	R_3	R_4
KY-193	OH	SO_3Na	Br	$>CHBrCH_2Br$
KY-194	OH	SO_3Na	Br	$>CH_2$
KY-270	$OCOCH_3$	SO_3Na	H	$>CH_2$
KY-272	$OCOCH_3$	SO_3Na	H	$>CHCO_2H$
KY-276	OCOEt	SO3Na	H	$>CH_2$
KY-277	COEtCl	SO_3Na	H	$>CH_2$
KY-280	OCH_3	SO_3Na	H	$>CH_2$
KY-281	$OCOC_3H_7$	SO_3Na	H	$>CH_2$
KY-284	OCH_3	SO3Na	H	$>CHCO_2H$
KY-285	$OCOCH_3$	SO_3Na	H	$>CH_2$
KY-288	OCOPr	SO3Na	H	$>CH_2$
KY-289	$OCOC_4H_9$	SO_3NH_4	H	>CH2
KY-290	OCOBu	SO_3Na	H	>H2
KY-291	$OCOC_5H_{11}$	SO_3NH_4	H	>CH2
KY-293	$OCOCH=CHCH_3$	SO_3NH_4	H	>CH2
KY-294	$OCO(CH_2)_4CO_2H$	SO_3NH_4	H	$>CH_2$
KY-307	$O(CH_2)_5CO_2H$	SO_3NH_4	H	>CH2
KY-346	OH	SO_3Na	H	$-CH_2N(CH_3)CH_2-$
KY-352	OH	$SO_3NHC_6H_{11}O_5$	H	$>CH_2$
KY-357	OH	$SO_2NHCH_2CO_2N$	H	$>CH_2$
KY-359	OH	SO_2NHOH	H	$>CH_2$
KY-395	OCH_3	SO_3Na	H	$-CH_2N(CH_3)CH_2-$
KY-397	OCH_3	SO_2NH_2	H	$>CH_2$
KY-398	OCH_3	OCH_3CO_2H	H	$>CH_2$
KY-399	OCH_3	$SO_2NHCH_2CO_2H$	H	$-CH_2N(CH_3)CH_2-$
Y-20	OH	SO_3Na	H	$-CH_2C_6H_2OCH_2-$
Y-34	OH	SO_3Na	H	$-CH_2C_6H_4CH_2-$
Y-66	OH	SO_3Na	H	$>CHCO_2H$
KYY-19	OH	$SO_2NHCH(CH_2)_2(CO_2H)_2$	H	$>CH_2$

Figures 3A and 3B illustrate two preferred synthetic methods for preparing macrocyclic chromotropic acid compounds. The method illustrated in Figure 3A involves cyclization of a chromotropic acid derivative (including chromotropic acid itself) with an aldehyde (RCHO) to form a macrocyclic compound, such as the tetramer shown in Figure 2, in which the chromotropic acid subunits are linked by R-substituted methylene groups, i.e., in which R_4 is >CHR (including ≥CR). This synthetic scheme provides a convenient method for constructing macrocyclic compounds having a variety of different bridge-methylene R groups, by carrying out the cyclization reaction in the presence of an aldehyde of the form RCHO.

For example, to construct a macrocyclic compound with a $-CH_2-$ bridge, such as the KY-1 compound (IV), chromotropic acid (III) is reacted with formaldehyde. Typical reaction conditions are given in Example 1A for the synthesis of KY-1. Similarly, KY-42 is prepared by cyclization with glyoxylic acid (Example 1C); KY-48, in the presence of glyceraldehyde; KY-85, in the presence of benzaldehyde; KY-97, in the presence of acrolein; and KY-110, in the presence of pyruvic aldehyde. It will be appreciated that a variety of other RCHO aldehydes having small alkyl, alkenyl, acid and other hydrocarbon R groups would be suitable. Further, the R bridge group may be further modified after the cyclization reaction. For example, KY-193 may be prepared by bromination of the KY-97 compound.

In the method illustrated in Figure 3B, cyclization of the chromotropic acid derivatives (III) is carried out by reaction with hexamethylenetetramine, to form a 3-atom chain bridge of the type $-CH_2N(CH_3)CH_2-$ (V). The cyclization reaction for the synthesis of KY-346 is given in Example 1J. The $-CH_2N(CH_3)CH_2-$ bridge may be modified, after the cyclization reaction, to form a variety of N-substituted bridges of the $-CH_2N(R')CH_2-$, where R' is one of a variety of small carbon-containing groups, according to known synthetic methods. Some of the bridges in the partially oxidized structure will have the form $=CHN(R')CH_2-$.

As noted above, the Figure-4A compound (VI) is representative of macrocyclic naphthalene having a cyclic bridge, in this case a phenyl bridge. The compound is formed by reacting chromotropic acid, in the presence of hydrochloric acid with 1,2-

benzenedimethanol in acetic acid, as detailed in Example 3. Similar methods can be employed to linked chromotropic acid subunits by other cyclic bridges, such as furan, pyran, pyrrole, and the like. Figures 4A and 4B show the non-oxidized (VI) and partially oxidized (VIII) forms of one compound).

For synthesis of macrocyclic compounds with selected R_1, R_2, and R_4 substituents, two general approaches are available. In one approach, the chromotropic acid derivative is modified after cyclization so that the cyclized product will either contain the selected R_1, R_2, and R_4 substituent, or contain a substituent which can be readily modified to the selected substituent. This approach is illustrated by the synthesis of KY-3, which has an SO_2NH_2 R_2 substituent, as detailed in Example 1B. Here cyclized chromotropic acid (VIII) is reacted first with chlorosulfonic acid, to form the corresponding $R_2 = SO_2Cl$ derivative (IX, Figure 5). The macrocyclic compound is then reacted with ammonia water to form the desired $R_2 = SO_2NH_2$ derivative (X, Figure 5).

A similar strategy was employed for the synthesis of KY-357 ($R_2 = SO_2NHCH_2CO_2H$) by final subunit reaction with glycine (XI, Figure 5), at basic pH.

Figure 6 (in combination with Fig. 5) illustrates the conversion of the sulfonate groups of cyclized chromotropic acid to sulfinate salts (XII) and sulfinate methyl esters (XIV). The first stage of the reaction involves formation of the corresponding sulfonyl chloride derivative (IX), as outlined above. This compound is then treated with sodium sulfite, to form the corresponding sulfinate

salt (XII). Reaction with dimethyl sulfate in the presence of sodium bicarbonate produces the corresponding sulfinate methyl ester (XIV).

Similarly, macrocyclic compounds with a variety of R_1 substituents may be prepared by modification of chromotropic acid after cyclization. In synthesizing KY-151, for example, ($R_1 = OCH_3$) cyclized chromotropic acid is reacted with dimethylsulfate under basic conditions, as detailed in Example 1F, to form the dimethylether of cyclized chromotropic acid. Similarly, in preparing KY-307 ($R_1 = O(CH_2)_5CO_2H$), cyclized chromotropic acid is first converted to the diether of hexanoic acid by initial reaction of cyclized chromotropic acid with 6-bromohexanoic acid under basic reaction conditions.

As a further example, in preparing compounds such as KY-272 and KY-294, in which R_1 has the form OCOR, the macrocyclic compound formed by cyclization of chromotropic acid is reacted with an acid chloride of the form RCOCl, under basic conditions, as detailed in Example 1J for the synthesis of KY-285.

In a second general approach, the selected substituent is formed on the subunit naphthalene rings by derivatization of the naphthalene subunit, with subsequent subunit cyclization to form the desired macrocycle. For the synthesis of KY-175 ($R_2 = SO_3CH_3$), chromotropic acid is reacted with sulfonylchloride, as above, to produce the corresponding $R_2 = SO_2Cl$ substituents. Further reaction with $NaOCH_3$ leads to the desired R_2 substituent. Reaction details are given in Example 1H.

It will be appreciated that the synthetic method for forming selected-substituent macrocyclic com-

pounds may include both prior derivatization of chromotropic acid and subsequent derivatization of the subunits after cyclization. For example, in forming KY-397 (R_1 = OCH_3, R_2 = SO_2NH_2), chromotropic acid subunits are first reacted at the R_1 positions, to form the dimethyl ether derivative as described above. After cyclization with formaldehyde, the compound is further derivatized at the R_2 position, also as described above, to convert the SO_3 group to the desired SO_2NH_2 substituent.

The KY compounds described above can be converted readily to a variety of sulfinate and sulfonate acids and salts. Salt and acid forms can be interchanged by passage over standard cation-exchange resins, to displace one cationic counter ion for another, according to well known methods. Thus, for example, several of the KY compounds shown in Table 1 are ammonium salts formed by cation exchange of protons in the presence of an ammonium salt, such as ammonium chloride. In addition, exposure of the macrocyclic compound to a variety of metal cations, such as the cations of Ca, Ba, Pt, Cu, Bi, Ge, Zn, La, Nd, Ni, Hf, or Pb, may produce both a metal salt and a metal chelate of the macrocyclic compound in which the metal is chelated at interior polar pocket in the compound.

The physical properties of several macrocyclic compounds prepared in accordance with the invention have been studied by absorption and mass spectrometry and by nuclear resonance spectroscopy (NMR), as detailed in Examples 1A, 1B, 1C, and 1J. These compounds include tetrameric macrocyclic compounds, such as indicated in Figure 2, or mixtures with predominantly tetrameric forms.

B. Calix(n)arene Compounds

Figure 7 shows the general structural formula of a calix(n)arene compound of the type that can be used in the method of the present invention. One exemplary compound of this type is shown in Figure 8, which is a tetramer of phenol parasulfonic acid subunits linked by methylene bridges (XV). As seen, the methylene bridges and the "interior" ring atoms (ring positions 2, 1, and 6) form a continuous chain having R_1 = OH groups attached at the 1 ring positions. The non-chain atoms (ring positions 3-5 on each substituent) have R_2 = sulfonic acid substituents on the 4 ring atoms.

Figure 9A illustrates a general method for forming calix(n)arene compounds. The precursor shown at the left (XVI) is a tert-butyl calix(n)arene, where n is the number of phenolic subunits (with para-position t-butyl substituents) in the macrocycle, and the bridge connections are methylene groups. t-butyl calixarenes with 4, 6, and 8, subunits are commercially available, and larger and uneven-numbered subunit calix(n)arenes can be prepared by standard purifying methods.

In the sulfonation reaction shown in Figure 9A, a t-butyl calixarene with a selected subunit number is treated with concentrated sulfuric acid, typically for about 4 to 5 hours at 75-85°C to effect substantially complete displacement of the 4-position t-butyl group by a sulfonic acid group. Details of the sulfonation reaction are given in Example 2A. The method has been used to produce the n=4 macrocycle compound shown in Figure 8, and related macrocycles with 6 and 8 phenol subunits.

A similar method is used for preparing a sulfonated calixarene with partially oxidized 1-position OH groups, as shown at 9B. Here the t-butyl calixarene starting material is treated with conc. sulfuric acid at a temperature above 100°C, preferably between 150-170°C. The reaction is effective to sulfonate the subunit rings and to partially oxidize the interior OH groups. As indicated in Figure 9B, partial oxidation can lead to a conjugated calix(n)arene structure (XVIII) in which the bridge contributes delocalized electrons. This conjugated structure is colored, and the development of a colored product can be used to monitor the course of the oxidation reaction. Details of the reaction are given in Example 2B.

It will be appreciated that the desired macrocycle can also be formed directly by reacting parasulfonic acid phenol (or precursors thereof) under suitable bridging conditions, such as described above for producing naphthalene-subunit macrocycles. This is illustrated by the reaction shown in Figure 12, for production of a macrocyle having carboxylic acid-containing bridge groups. In this method, phenol parasulfonic acid is reacted with glyoxylic acid, under conditions similar to those described in Example 1C, to form the cyclized structure shown (XXII). A synthesis of XXII is described in Example 2F.

The calix(n)arene compounds formed as above can be modified, according to general procedures outlined in Section IIA above, to achieve selected R_1 groups, modified sulfonyl groups, and/or addition of R_2 groups. The range of R_1 and R_2 substitutents is substantially the same as that discussed in Section

IIA above. Figures 10, 11, and 13 illustrate various reaction methods for modifying the R_1 or R_4 group of an already formed macrocycle. In Figure 10, the sulfonated structure shown in Figure 8 is treated with acetic anhydride, to form an O-acetyl R_1 group. Details of the reaction are given in Example 2C.

Example 2G describes a similar reaction scheme for forming a toluene sulfonic acid ester at the R_1 position.

Figure 11 illustrates a general method for forming sulfonamides, such as glycylsulfonamide (XXI) of the Figure 8 compound. Analogous to the reactions described with respect to Figure 5, the sulfonated phenyl calix(n)arene compound (XVII) is treated with chlorosulfonic acid, to form the corresponding sulfonyl chloride analog (XX). Further reaction with a selected amine, in this case glycine, gives the desired sulfonamide. Reaction details are given in Example 2D for the synthesis of the $R_2 = SO_2NH_2$ compound and in Example 2E, for the synthesis of the glycyl sulfonamide compound.

Figure 13 depicts a general non-exclusive synthetic method for a net substitution of R_1 = OH by R_1 = carbon moieties. In Example 2H, the reactions detail a process from which a substrate (R_1 = OH, R_2 = tert-butyl, $R_4 = CH_2$, n = 4) affords an intermediate (R_1 = CN, R_2 = tert-butyl, $R_4 = CH_2$, n = 4). Further modification then provides the product ($R_1 = CO_2H$, $R_2 = SO_3H$, $R_4 = CH_2$, n = 4).

It will be appreciated that substituent modifications at the R_1 site can be selectively carried out at OH sites in a partially oxidized macrocycle, such as the structure shown at Figure 9B. That is, reactions which are specific for ring OH

groups will leave the =O group intact, thus providing a mixed R_1 group containing =O groups.

The R_3 is generally H, but may be an uncharged or negatively charged substituent, similar to the R_3 group described in Section IIA above.

The R_4 bridge linking the chromotropic acid derivative subunits is preferably of the form >CHR or ≥ CR, where R is H or a small carbon-containing group, such as lower alkyl, alkenyl, ketone, or carboxylic acid group, or aryl group, as noted above, or of the form $-CH_2NR'CH_2-$, where R' is similarly H or a small carbon-containing group, such as a lower alkyl group. Alternatively, the bridges in the macrocycle may be ring structures, including aryl ring structures, analogous to the dimeric macrocycle shown in Figure 4.

Also as above, the number of subunits may vary from four (e.g., Figure 8 structure) to 8, with macrocycles containing 4, 6 and 8 subunits being preferred. In the reaction schemes described below, the macrocycle formed may include mixtures of compounds with different subunit numbers (n) values, e.g., a dominant n=4 structure (4 subunits) plus additional structures containing 5-8 subunits.

Representative calix(n)arene compounds which have been synthesized and tested for anti-viral activity are identified by their R_1, R_2, and R_4 substituents in Table 2 below. The KY and Y number in the lefthand column in the table refers to the analog designation of the corresponding compound, as in Table 1. Compounds which are partially oxidized at the R_1 position, and which may have both saturated and unsaturated bridge methylene carbon groups are indicated in column 2 of the table.

Table 2

Compound	R_1	R_2	R_4	n
Y-1	OH	SO_3	$-CH_2-$	8
KY-226	O/OH	SO_3	$-CH_2/=CH-$	8
Y-49	OH	SO_3	$-CH_2-$	4
KY-225	O/OH	SO_3	$-CH_2/=CH-$	4
Y-77	OH	SO_3	$-CH_2$	6
Y-48	O/OH	SO_3	$-CH_2/=CH-$	6
KY-268	O/OH	SO_3	$-CH_2/=CH-$	3
KY-269	O/CO_2CH_3	SO_3	$-CH_2/=CH-$	4
KY-271	O/CO_2CH_3	SO3	$-CH_2/=CH-$	3
Y-78	O/OH	SO_2NH_2	$-CH_2-$	8
Y-100	O/OH	SO_2OCH_3	$-CH_2-$	8

The compounds shown in Table 2, and R-group combinations thereof, described above can be converted readily to a variety of sulfonic acid or sulfonate salts, by reaction in acid or in the presence of a suitable salt, according to well known methods, as described above.

C. Macrocyclic Compounds with Sulfonate, Sulfinate, Phosphonate, and Carboxylate Groups

One general class of compounds which are useful in inhibiting infection by enveloped viruses, in accordance with the present invention, are calix(n)arene compounds in which the ring position meta to the bridge attachments, i.e., 4-position carrying substituent R_2 in Fig 7, is substituted with a negatively charged substitutent having a terminal sulfonate, sulfinate, phosphonate, or carboxylate group.

Methods for preparing calix(n)arene compounds in which a sulfonate group is carried at the ring 4 position are given in Examples 2A, 2B, and 2C, including compounds with different substitutions at the ring 1 position. Compounds having sulfonamide

group, including a group which terminates with an end terminal carboxyl group are given in Examples 2D and 2E, respectively.

Fig. 14 illustrates a method for converting a t-butyl calix(n)arene (XXV) to the unsubstituted compound (XXVI), which can be used as a starting material for some of the syntheses described below. Details of the reaction are given in Example 2J.

Fig. 15 shows the conversion of a calix(n)arene (XXVII) carrying a p-acetyl group to the corresponding calix(n)arene with a p-carboxyl group (XXVIII). Details are given in Example 2K.

To form the p-carboxyethyl compound shown at XXXI in Fig. 16, compound XXVI produced above is converted to the corresponding (dimethylamino)methyl compound (XXIX) by reaction with dimethylamine and formaldehyde. This compound is then taken to the corresponding cyanomethyl compound (XXX), which when heated in acid, is converted to the desired carboxymethyl compound (XXXI). Details are given in Example 2L.

Fig. 17 illustrates the synthesis of a carboxyethyl calix(n)arene (XXXIII). Here the intermediate (XXIX) from above (Fig. 16) is treated sequentially with MeI and the sodium salt of diethylmalonate to give the diethylmalonylmethyl compound (XXXII). Heating in acid gives the desired compound XXXIII. Details are given in Example 2M.

Fig. 18 illustrates the synthesis of a p-phosphonate calix(n)arene (XXXVI). In this synthesis, compound XXVI from above is iodinated and then reacted with diethylphosphite to give the diethylphosphonate compound (XXXV). Refluxing in acid gives the desired compound XXXVI. Details are given in Example 2N.

The synthesis of a phosphonomethyl calix(n)arene (XXXIX) is shown in Fig. 19. As seen, compound XXVI from above is chloromethylated (compound XXXVII), and further reaction with triethylphosphite gives a diethylphosphonyl ester compound (XXXVIII). Heating in acid gives the desired phosphonomethyl compound. Details are given in Example 2O. Note that the chloromethyl intermediate is also useful in synthesis of the sulfonylmethyl calix(n)arene analog.

The synthesis of a p-2-bromoethyl compound useful in the synthesis of a phosphonoethyl or sulfonylethyl calix(n)arene is outlined in Fig. 20, with details given in Example 2P. With reference to the figure, compound XXVI from above is allylated at the phenol hydroxyl (compound XL), and heated to give the rearrangement product XLI. Tosylation serves to protect the phenyl hydroxyl position (compound XLII), allowing conversion to the p-hydroxyethyl derivative (XLIII). Further reaction with triphenylphosphine dibromide gives the desired p-bromoethyl XLIV compound.

The p-bromoethyl calix(n)arene (XLIV) is used in the synthesis of the p-phosphonoethyl compound (XLVI), by a reaction sequence which is analogous to that shown in Fig. 19, described above. Details of the reaction scheme shown in Fig. 21 are given in Example 2Q.

The intermediate p-chloromethyl calix(n)arene (compound XXXVII) used above can also be used in the synthesis of a p-sulfonylmethyl calix(n)arene (XLVII), as shown in Fig. 22, with details provided in Example 2R.

Similarly, the p-2-bromoethyl intermediate (XLIV) described above can be used in the synthesis

of the p-sulfonylethyl calix(n)arene (XLIX) as shown in Fig. 23, with details given in Example 2S.

It will be appreciated that when desired, sulfonate groups can be converted to sulfinate groups by the methodology illustrated in Fig. 6 and exemplified in Example 1D.

The foregoing synthetic methods can be used to produce calix(n)arene compound having at the position meta to the bridge positions (the C4 ring positions), negatively charged substituents which terminate with sulfonate, sulfinate, phosphonate, and carboxylate groups. The syntheses outlined show both direct acid-group attachment to the rings, or attachment through alkyl linkages, such as methyl and ethyl linkages. It will be appreciated from the discussion above how acid groups linked to the rings through longer alkyl groups can be prepared. Also, as detailed above, the acid groups can be converted to the corresponding salts.

It will also be appreciated how a variety of esters and amides of the terminal acid groups in the macrocyclic compounds of the invention (e.g., calixarenes) can be prepared. Such derivative can be used as "pro-drugs", where an ester or amide is converted *in vivo* to the corresponding negatively charged acid group by enzyme-catalyzed hydrolysis (e.g., by an esterase).

Generally, the acid esters of carboxylic acid and sulfonic acid can be prepared by standard esterification reactions in which the acid is converted to, for example, an acid chloride, then reacted with an alcohol, such as an alkyl alcohol. The amides of carboxylic and sulfonic acid can similarly be formed by reaction of the acid chloride with an amine, such as an alkyl amine. Preferred

esters include aryl and lower alkyl carbonate esters, such as n-butyl alkyl group. Preferred amides include amides of lower alkyl groups.

The conversion of a phosphonic acid calix(n)arene to a corresponding ester or amide likewise follows conventional phosphonate esterification or amidation reaction methods. One method for generating a diethylphosphonyl ester has been described above with reference to Fig. 21.

In addition to the polar substituents at the C4 position in the calix(n)arene rings, the present invention contemplates, for use in the method of the invention, calix(n)arene compounds which are substituted at other ring positions and at the bridge positions in the macrocycle. For example, the C3 and/or C5 ring positions may be substituted with halogens such as F of Cl. Also as described above with respect to several of the naphthyl-ring macrocycles, substitutions at the "inner" ring positions (the C1 ring positions in calix(n)arene) are compatible with anti-viral activity. Also as described above, substitutions at the bridge positions in naphthyl-ring macrocycles are compatible with activity.

Figs. 24 and 25 illustrate one method of attaching carboxyl groups to the bridge methylene in calix(n)arene. In this method, the hydroxyl group of p-t-butyl calix(n)arene (XXV) is acetylated (L), and the product is oxidized at the bridge methylene, to give the bridge ketone (LI). Reduction with sodium borohydride, and subsequent reaction with thionyl chloride yield the compound LIII which is chlorinated at the methylene bridge. Details are given in Examples 2T and 2U.

With reference to Fig. 25, the compound LIII is cyanylated, then treated with acid to form the carboxylic acid group at the bridge methylene. The resulting compound LV can be de-t-butylated by treatment with aluminum chloride to give the bridge-carboxylated calix(n)arene shown at LVI. Alternatively, compound LV may be sulfonated, at the C4 ring position, by treatment with sulfuric acid, as above. It will be appreciated that similar methods, but involving initial protection of the bridge carboxyl and/or ring hydroxyl groups, can be used to form corresponding p-phosphonic acid or p-carboxylic acid calix(n)arenes.

Using compound LIII from above, a variety of bridge substitutions can be produced, by the method outlined in Fig. 26, using a suitable cuprate reagent as illustrated at the left in the figure. The reaction at the right in the figure shows how a calix(n)arene LIII can be converted to a compound having a carboxymethyl group attached to the bridge methylene group. Details of this reaction sequence are given in Examples 2W and 2X. The final reaction product (LVIII) can be p-sulfonated or derivatized with other acid groups at the para position as above.

Fig. 27 shows a variety of derivatization reactions involving calix(n)arenes and propane-1,3-sulfone. The reactions are effective to add alkylsulfonate groups at ring hydroxyl positions, as shown. This method provides an alternative approach for producing calix(n)arenes with ring-attached sulfonic acid groups. Exemplary reaction conditions are given in Examples 2Y and 2Z.

Finally, Fig. 28 shows the preparation of a mixed macrocyle containing alternating phenyl and

naphthyl groups. The reaction method is described in Example 3B.

III. Inhibition of Virus Infectivity

This section examines the ability of compositions containing a macrocyclic compound to inhibit infection by a variety of enveloped viruses. The enveloped viruses which were examined are the herpes viruses, herpes simplex virus-1 (HSV-1) and herpes simplex virus-2 (HSV-2), which are double-stranded DNA viruses (Roizman); human immunodeficiency virus (HIV), an RNA retrovirus (Popovic; Barre-Simoussi); and influenza A and B and respiratory syncytial viruses (RSV), all RNA viruses (Chanock).

For comparative purposes, selected non-enveloped viruses, including adenovirus, a double-stranded DNA virus (Rowe; Hilleman), and rhinovirus, a single-strand RNA virus (Dick), were examined. Typically, inhibition of virus infectivity was measured by the extent of inhibition of cytopathic effects detectable in infected cultured cells. Inhibition of HSV-1 and HSV-2 infectivity in cultured cells was also shown by inhibition of virus binding to infectable cells, and inhibition of viral plaque formation in infected cells, as described below.

In addition, a large number of representative aryl macrocyclic compounds (including those shown in Tables 1 and 2) were examined for toxicity in cell culture, using a panel of human cell lines, as detailed in Example 4. Briefly, the selected KY- or Y- compound was added to cell cultures at a final concentration of 5, 10, 25, 50, or 100 μg/ml. Three days later the cells were washed to remove drug, and stained with a vital stain, to determine the

percentage of dead cells in each culture. The IC_{50} drug concentration, i.e., value concentration of drug which produced 50% cell death, was 50μg/ml for KY-143, KY-151, and KY-163, and 100μg/ml or greater for all of the other KY compounds tested. For KY-1, which has a molecular weight of 1404 daltons, a drug concentration of 100 μg/ml is equivalent to about 66 μM.

A. Inhibition of HSV Infectivity: Naphthalene-Subunit Compounds

Several compositions containing one of the compounds in Tables 1 and 2 were tested for inhibition of cytopathic effects (CPE's) in cultured, HSV-infected cells. In the method reported in Example 5, Vero cells were infected with HSV-1 or HSV-2 and allowed to grow in culture until cytopathic effects were clearly visible. In the absence of infection, the cells form an even monolayer of fibroblast-like cells. With HSV infection, a cytopathic effect characterized by round cells in suspension is clearly evident after 24 hours, followed by clumping and lysis of infected cells after 24-72 hours.

In the drug inhibition study reported in Example 5, cells were exposed to HSV-1 or HSV-2 virus and, at the same time, to a selected aryl macrocyclic compound, at a final drug concentration of 10 μg/ml. Twenty-four hours later the cells were examined for cytopathic effect. If a clear cytopathic effect was not observed with 10μg/ml of the drug, the study was repeated at a drug concentration of 20 μg/ml for some compounds.

Table 3 below lists 50 naphthalene-subunit macrocycles which were tested in this assay. A "+" symbol in the second column indicates that the

compound was effective in inhibiting cytopathic effects at 10 or 20 μg/ml. A "-" symbol indicates that CPE was observed at 10 or 20 μg/ml.

Table 3

Compound	CPE 10,20 μg/ml	HSV-1 IC_{50} (μg/ml)	HSV-2 IC_{50} (μg/ml)
KY-1	+	2.7	1.7
KY-3	+	2.4	2,5
KY-42	+	1	3
KY-48	-	N'	N
KY-85	-	N	N
KY-97	+	N	N
KY-110	-	N	N
KY-121	+	1.5	1.8
KY-123	+	1.5	1.5
KY-129	+	1	1
KY-143	-	N	N
KY-147	-	N	N
KY-148	-	N	N
KY-151	+	1.25	1.8
KY-158	-	N	N
KY-171	+	2.5	3
KY-175	-	N	N
KY-176	N	N	N
KY-193	GC	N	N
KY-194	+	1	1
KY-280	+	2	2
KY-272	+	N	N
KY-276	+	1.3	1.2
KY-277	+	1	1.2
KY-280	+	1.1	1
KY-281	+	0.5	1.5
KY-284	+	1	1.6
KY-285	+	1	1.5
KY-286	+	2	2
KY-288	+	1.7	2
KY-289	+	2.2	1.7
KY-290	+	1.2	1.3
KY-291	+	1.4	2
KY-293	+	1.9	2.7
KY-294	+	1	2.2
KY-301	+	1	1
KY-307	+	.8	2
KY-308	+	.9	1.2
KY-345	+	5	6.7
KY-346	+	4.4	6.2

Table 3

Compound	CPE 10,20 μg/ml	HSV-1 IC_{50} (μg/ml)	HSV-2 IC_{50} (μg/ml)
KY-352	+	3.4	4.1
KY-357	+	4	3.3
KY-359	+	5.75	4.2
KY-376	+	2.7	1
KY-395	-	N	9
Y-4	+	5.5	6.4
Y-14	+	2.5	3.5
Y-20	+	5	3.2
Y-34	+	2.5	2
Y-66	+	N	N

[1] N, no inhibition of CPE observed at highest concentration tested, or insufficient inhibition observed to predict IC_{50}.

The compounds used in Table 3 were further tested for activity against HSV infection in a plaque reduction assay, as detailed in Example 6. Here Vero cells, after overnight incubation, were exposed to serial dilutions of KY compound, from 0.625-10 μg/ml, and HSV-1 or HSV-2 virus for two hours. After washing to remove drug and extracellular virus, the cells were further incubated for 2 days, then stained and counted for plaque formation. Percent inhibition was determined by dividing plaques produced by total number of plaques in infected, untreated controls. From the concentration effect curve of plaque inhibition (expressed as percent of control), the concentration of compound required to produce 50% plaque reduction, IC_{50}, was determined. The IC_{50} values for infection by HSV-1 and HSV-2 infection is given in the right-hand columns in Table 3.

With reference to the compound structures given in Table 1, the following R-group features can be identified as contributing to low activity (no protection of cells from CPE effect seen at 10-20

μg/ml: in KY-48, KY-49 and KY-110, a bulky side chain in the methylene bridge; in KY-143, an OH R_3 group; in KY-147 and KY-148, a sulfonamide with a non-polar alkyl group at the R_2 position; in KY-158 and KY-175, a sulfinate ester or sulfonate ester with a non-polar alkyl group at the R_2 position; and in KY-395, a trimethylamine bridge in combination with a methyl ether substituent at the R_1 position. The "GC" symbol for KY-193 means that some giant cells were formed, indicating partial inhibitory activity.

Looking now at the compounds which give complete inhibition of CPE at 10-20 μg/ml, the following R-group structures can be identified as preferred radicals:

The R_1 position contains OH, including combinations of OH and =O groups; alkyl and aryl esters, including combination of such esters and =O; and alkyl ethers, including combinations of such ethers and =O.

The optimal radicals at the R_2 position are sulfonic acid or sulfonic acid salts, sulfinic acid and salts thereof, and sulfonamides with polar amine groups, such as NH_2, NHOH, N-glycosides (KY-352), and amino acids.

The preferred radicals at the R_3 position are H or Br.

The optimal bridge linkage groups are substituted and unsubstituted methylenes, where the R group is not a bulky alkyl group, and is preferably a carboxylic acid group.

As a further guide to R-group selection, compounds having an ED_{50} value of $\leq$ 1μg/ml for at least one of the two HSV tests have one of the following R-group characteristics:

Compounds whose R_1 groups are lower-alkyl ethers or esters, or contain a terminal carboxylic acid group are typically most active, especially in combination with =O groups at other R_1 sites in the compound.

The R_2 groups are sulfonic acid or sulfonic acid salts or sulfonamides with a terminal carboxylic acid. This feature indicates that an R_2 position acid group favors high activity.

The R_4 bridge is methylene or a methylene carrying a carboxylic acid group.

The ability of selected naphthalene-subunit compounds to inhibit HSV-1 and HSV-2 viral yields at selected drug concentrations up to 10 μg/ml was assessed in the viral inhibition assay described in Example 7. Briefly, cultured Hela cells were exposed to serially diluted KY compound and virus, allowed to grow for 24 hours, then freeze/thawed 3 times to release virus particles. Vero cells were infected serial dilutions of the viral lysates were assayed for plaque counts as described in Example 6. The drop in viral yield, as a function of drug concentration, is plotted in Figures 29A and 29B for compounds KY-1 and KY-42 respectively. The dose dependent drop in viral yield was between about 3-5 orders of magnitude, depending on drug and virus. The degree of inhibition of viral yield was generally greater for HSV-1 than for HSV-2. Similar results were observed with several other KY compounds.

B. Inhibition of HSV Activity: Calix(n)arene Compounds

A similar study of anti-HSV activities was carried out with several of the calix(n)arene compounds listed in Table 2 above, with the results

shown in Table 4. As seen from column 2 of the table, all of the compounds which were tested gave inhibition of CPE at 10-20 μg/ml. Inhibition activity of the tested compounds against HSV-I and HSV-2 in the plaque reduction assay is shown in the two righthand columns in the table (given as IC_{50} in μg/ml).

Table 4

Compound	CPE 10,20 μg/ml	HSV-1 IC_{50} (μg/ml)	HSV-2 IC_{50} (μg/ml)
Y-1	+	7	7.2
KY-226	+	1.6	3
Y-49	+	5	10
KY-225	+	1.8	1.8
Y-77	+	8	11
Y-48	+	1.5	1
KY-268	+	4.4	1.5
KY-269	+	2.4	2.3
KY-271	+	4.2	2.4

The highest activities observed for the phenyl-subunit compounds are comparable to the highest activities seen with the naphthyl compounds, e.g., from about 1-3 μg/ml IC_{50} values.

The most active compounds, Y-226 (n=8), Y-48 (n=6), and Y-225 (n=4) all have partially oxidized R_1 OH/=O groups, and each partially oxidized compound is substantially more active than its corresponding non-oxidized analog.

The partially oxidized n=3 compound, KY-268, is somewhat less active than its n=4, 6, and 8 analogs. Among the non-oxidized compounds, the *n*=8 compound, Y-1, is somewhat more active than the corresponding n=4 and n=6 compounds.

Addition of acetyl groups at the R_1 position produces little change in the activities of partially oxidized compounds, also consistent with the results observed with the naphthalene-subunit compounds, addition of alkyl esters at the R_1 positions gave activities comparable to the partially oxidized analog.

As a general guide to optimizing compound activity in the phenyl-subunit compounds, the same rules discussed above generally apply. Thus, for example, highest activity is expected when the R_2 group terminates in a negatively charged group, such as a sulfonic acid. More generally, the present invention contemplates, for use in treatment of infection by enveloped virus, a calix(n)arene compound which whose R_2 group is a polar substituent which terminates in a sulfonic acid, phosphonic acid, or carboxylic acid group, including esters and amides of these acids which can be converted to the corresponding acid by hydrolytic cleavage *in vivo*. For use in inhibiting infection by a sexually transmitted enveloped virus, the present invention contemplates a macrocyclic compound whose R_2 groups are negatively charged substituent which terminates in a sulfonate, sulfinate, phosphonate, or carboxylate group.

The compounds may also include esters and amides of the negatively charged groups which can be converted to acid groups by hydrolytic cleavage *in vivo*. A variety of esters and amides of sulfonic acid, phosphonic acid, and carboxylic acid have been shown to undergo hydrolytic cleavage *in vivo* to the corresponding acids (Svensson, 1988, 1991; Stella; Bundgaard) including esters and amides of lower alkyl

groups. Methods for preparing a variety of exemplary compounds of this type are described in Section II above.

The calix(n)arene compounds having terminal acid groups (or groups cleavable to terminal acid groups) may be substituted at other ring and bridge positions, as indicated in Section IIB and IIC above. In one preferred embodiment, the R_i substituent is an OH group, or, where the compound is partially oxidized, a combination of OH and =O groups.

C. Comparison of Anti-HSV Compounds

The inhibitory effect of KY-1 against drug-resistant strains of HSV-1 and HSV-2 was compared with several anti-viral agents which have been used in treating HSV infection. The compounds tested were the nucleoside analogs acyclovir (ACV), ganciclovir (DHPG), phosphonoformate (PFA), and phosphomethoxyethyladenine (PMEA). Inhibition of viral yield was determined, as above, by infecting Hela cells in the presence of wild type or drug-resistant strains of HSV-1 or HSV-2, and serial dilutions of a selected anti-viral compound, and infecting Vero cells with serial dilutions of the Hela cell lysate, as above. Details of the inhibition study are given in Example 8.

The ID_{90} concentration (which effects 90% inhibition of viral yield) is given in Table 5. The KOS (HSV-1) and 333 (HSV-2) are wild type viruses; the KOS(PMEA)' and KOS(PFA)' are drug-resistant HSV-1 strains having a DNA polymerase mutation. The 333(DHPG) strain is a drug-resistant HSV-2 strain having a thymidine kinase mutation. With the exception of DHPG as an inhibitor of drug-resistant

strains of HSV-1, and PMEA as an inhibitor of drug-resistant strains of HSV-2, all of the nucleoside analogs were at least about 20 times less active against drug-resistant strains than wildtype strains of either HSV-1 or HSV-2, as measured by drug concentration required to inhibit yield. By contrast, the aryl macrocyclic compound showed substantially the same specific activity against drug-resistant strains of HSV-1 and HSV-2 as against wildtype strains.

Table 5

			Drug Tested (ID_{90}) #				
Virus	Strain/ drug selection	Mutation Locus	KY-1 (ug/ml)	ACV (uM)	DHPG (uM)	PFA (uM)	PMEA (uM)
HSV-1	KOS	None	1.9	14	2	180	100
	KOS(PMEA)'	DNA pol	2.6	380	NT	3000	>2000
	KOS (PFA)'	DNA pol	4.3	100	1	>1000	>1000
HSV-2	333	None	3.2	=10	2	150	155
	333 (DHPG)'	TK	3.7	>100	215	NT	120

The data demonstrate that aryl macrocyclic compounds are effective against drug-resistant HSV strains at drug concentrations comparable to those which are effective against wild type virus strains. By contrast, and with the exception of DHPG as an inhibitor of HSV-1 strains, both drug-resistant strains showed a significant resistance to ACV, DHPG, PFA, and PMEA, as evidenced by the severalfold greater ID_{90} drug concentrations required for virus inhibition.

D. Inhibition of RSV and Influenza A Virus Infectivity

Representative macrocyclic compounds from Table 1 were tested for inhibition of cytopathic effects in

cultured MDCK or HEp2 cells after infection by Influenza A virus (A/Taiwan strain) or RSV virus. In the method of inhibiting virus infectivity by influenza A, MDCK cells were infected with the virus, and the cells were allowed to grow in culture until cytopathic effects were clearly visible. In the absence of infection, the cells form an even monolayer of fibroblast-like cells. With virus infection, a cytopathic effect characterized by cell clumping is observed. For each compound tested, drug concentrations of 0.1, 1, 10, 25, and 100 μg/ml were added to cultured cells at the time of virus infection, as detailed in Examples 9 and 11. Twenty-four hours later the cells were examined for percent clumping, based on the percent of clumped cells of total cell particles in a given view field. The inhibition of clumping was plotted as a function of drug concentration, to determine the dose effective to produce a 50% reduction in the percent clumped cells, measured with respect to control (no drug treatment). The measured ED_{50} values are given in Table 6 below.

A similar method was employed to determine the ED_{50} of RSV inhibition of cytopathic effect (cell clumping) in HEp2 cells, with the results shown in Table 6. Details are given in Example 9.

Table 6

	ED_{50} (μg/ml)	
Compound	Influenza A (Taiwan)	RSV

KY-1	>188	0.19
KY-3	6	0.75
KY-42	94	1.50
KY-47	94	>250
KY-85	>250	1
KY-97	5	0.5
KY-110	>250	4
KY-123	31.3	1
KY-151	>94	1.5
KY-193	5.0	0.8
KY-194	7.9	0.5

In general, RSV was significantly more sensitive to compound inhibition than the Influenza A/Taiwan virus. Highest-IAV activity was seen with a sulfonamide with polar amine (SO_2NH_2) at the R_2 position, and with selected methylene bridge groups. Relatively high anti-RSV activity was seen with all compounds except KY-47.

E. Inhibition of HIV Infectivity: Naphthalene-Subunit Compounds

Representative macrocyclic compounds from Table 1 were tested for inhibition of cytopathic effects in cells infected with one of two HTLV-III strains, HTLV-III_B and RF-II strains, as described in Example 12. Briefly, cells chronically infected with HTLV-III_B or RF-II HIV strains were incubated in the presence of serial dilutions of the selected KY compound, then further cocultured with indicator cells. The extent of syncytia formation was scored under phase microscopy. The concentration ($\mu g/mL$) effective to produce complete inhibition of syncytia formation, ED_{100}, is shown in Table 7 for the two HIV strains. The "N" means that the compound was not tested for that virus.

Table 7

Inhibition of Syncytia Formation

Compound	HIV-HXB ED_{100}	HIV-RF-II ED_{100}
KY-1	8	N
KY-3	16	N
KY-42	8	N
KY-48	250	N
KY-85	32	N
KY-97	32	N
KY-110	63	N
KY-121	16	16
KY-123	16	16
KY-129	16	8
KY-143	250	125
KY-147	250	250
KY-148	250	N
KY-151	32	125
KY-158	500	7500
KY-171	125	250
KY-175	63	250
KY-176	125	250
KY-193	63	500
KY-194	63	125
KY-270	16	32
KY-272	63	250
KY-276	16	32
KY-277	16	32
KY-280	16	32
KY-281	16	32
KY-284	16	32
KY-285	16	32
KY-286	16	32
KY-288	8	16
KY-289	16	32
KY-290	16	32
KY-291	16	32
KY-293	16	63
KY-294	16	16
KY-301	8	8
KY-307	8	32
KY-308	8	32
KY-345	63	125
KY-346	16	32

Table 7

Inhibition of Syncytia Formation

Compound	HIV-HXB ED_{100}	HIV-RF-II ED_{100}
KY-352	32	125
KY-357	32	63
KY-359	32	63
KY-376	8	16
KY-395		
Y-4	8	125
Y-14	16	32
Y-20	4	16
Y-34	N	N
Y-66	N	N

As seen from these results there is a general correlation between anti-viral activity against the two strains; that is, compounds which are most active against the HTLV-III_B strains are also most active against the RF-11 strain.

With reference to the compound structures given in Table 1, the following R-group features can be identified as contributing to sub-optimal activity (ED_{50} values $\geq$ 63 µg/ml for both strains): in KY-48, a bulky side chain in the methylene bridge; in KY-110, a methyl ketone group in the bridge; in KY-143, an OH R_3 group; in KY-147 and KY-148, a sulfonamide with a non-polar alkyl group at the R_2 position; in KY-158 and KY-175, a sulfonate ester or sulfinate ester having a non-polar alkyl group, at the R_2 position; and in KY-272, a methyl ester at the R_1 position combined with a acetyl-group bridge. These features are substantially the same as those which gave reduced activity against HSV viral infectivity, i.e., showed no inhibitory effect on CPE at 10-20 µg/ml.

Similarly, those factors which promote high activity against HSV activity are in general the same

as those which give highest activity against HIV infectivity. These factors include: the groups at the R_1 position are OH, including combinations of OH and =O groups (partial oxidation); alkyl and aryl esters, including combination of such esters and =O; and alkyl ethers, including combinations of such ethers and =O.

The preferred radicals at the R_2 position are sulfonic acid or sulfonic acid salts, sulfinic acid and salts thereof, and sulfonamides with polar amine groups, such as NH_2, NHOH, N-glycosides (KY-352), and amino acids, with sulfonic acid. In particular, high activity was seen with sulfonic acid, sulfonate salts, and sulfonamides having a terminal carboxylic acid group.

The optimal radicals at the R_3 position is H, with both OH and Br giving reduced activity.

The bridge groups are preferably substituted and unsubstituted methylenes, where for a >CHR or $\geq$CHR bridge, the R group is not a bulky alkyl group.

As a further guide to R-group selection, compounds having an ED_{50} value of $\leq$ 1μg/ml for at least one of the two HSV tests have one of the following R-group characteristics:

Compounds whose R_1 groups are lower-alkyl ethers or esters, or contain a terminal carboxylic acid group are typically most active, especially in combination with =O groups at other R_1 sites in the compound.

The R_2 groups are sulfonic acid or sulfonic acid salts or sulfonamides with a terminal carboxylic acid. This feature indicates that an R_2 position acid group favors high activity.

The R_4 bridge is methylene or a methylene carrying a carboxylic acid group.

These preferred R-groups are intended to provide guidance in the selection of R groups at the R_1-R_4 positions, for optimizing compound efficiency.

F. Inhibition of HIV Infectivity: Calix(n)arene Compounds

Representative calix(n)arene compounds from Table 2 were tested for inhibition of cytopathic effects in cells infected with one of two HTLV-III strains, HTLV-III_B and RF-II, as described in Example 12, and in the subsection above. The IC_{50} values measured for the HXB and RS-11 strains of HIV are given in units of μg/ml in Table 8 below.

Table 8

Compound	HIV-HXB IC_{50}	HIV-RF-11 IC_{50}
Y-1	16	N
KY-226	16	250
Y-49	N	N
KY-225	32	125
Y-77	N	N
Y-48	N	N
KY-268	32	32
KY-269	32	32
KY-271	32	63

Interestingly, the Y-1 compound and KY-226 (the corresponding partially oxidized analog) have comparable activities against the HXB strain, in contrast to the significantly higher activity of KY-226 seen against HSV viruses. All of the other compounds tested have partially oxidized R_1 =O groups, and all compounds give comparable activity.

IV. Specificity Toward Enveloped Viruses

This section examines the specificity of the viral-inhibition method to enveloped viruses. The studies reported in subsection A show that the macrocyclic compounds used in the method act, at least in part, by binding selectively to viral envelope proteins, and that this binding blocks virus attachment to infectable cells, thereby inhibiting virus infectivity. These studies are detailed in U.S. patent application Serial No. 647,720, filed January 29, 1991, now U.S. Patent No. 5,196,452. Subsection B examines the inhibitory effect of the macrocyclic on non-enveloped viruses.

A. Mechanism of Viral-Infection Inhibition

In one study, the ability of a macrocyclic compound to block HSV binding to infectable cells was examined as described in Example 14. Briefly, Vero cells were exposed to radiolabeled HSV-1 or HSV-2 in the absence of KY compound or in the presence of 10 μg/ml KY-1, and binding of the virus at times up to 4 hours after exposure to the virus was measured. Figure 30 shows a plot of virus (radiolabel) binding to cells over the four-hour incubation period. In the absence of drug, the amount of bound virus increased steadily over two hours, and slightly from 2-4 hours. By contrast, virus binding to cells peaked at about 1/2 hour in the presence of drug, presumably reflecting the time during which the binding events effective to block virus binding to the cells are equilibrating.

In a second study, the effect of compound when administered prior to, during, or after cell infection by HSV-1 was examined as described in Example 15. In these studies, cells were exposed to

one of a series of increasing KY-2 concentrations, and the extent of infection was measured by number of plaques observed 24 hours after infection. The reduction in plaque formation, expressed as a percent of control, is shown in Figure 31 for cells treated with drug prior to (solid rectangles), during (closed circles), and after (open rectangles). Virus inhibition was seen most significantly when the cells were treated with drug during exposure to virus, indicating that virus inhibition occurs at the period of virus binding to and entry into infectable cells.

In a third study, purified HSV-1 virus suspensions were incubated with KY-1 or the sodium salt thereof, or a control solution for 1 hour, then serially diluted to drug concentrations between 10^{1} to 10^{-4} µg/ml as described in Example 16. Addition of the serially diluted virus suspensions gave the plaque counts, measured in duplicate, shown in Table 9. The "X" symbol in the table indicates plaques too numerous to count. The results of the study demonstrate that inhibition of HSV infection by KY compounds is due, at least in part, to binding of drug to HSV particles. Further, complete virus inhibition was seen at drug final drug concentration of 10^{-2} to 10^{-4} µg/ml (which are much lower than those needed to inhibit HSV in Vero cell culture). It can be concluded that the drug-binding/inactivation of the virus is effectively irreversible, i.e., not reversed by high dilution effects.

Table 9

KY Compounds	Initial Viral Input (pfu/cell)	Plaque Number after Serial 10-time Dilutions					
		1	10^1	10^2	10^3	10^4	10^5
Control (media only)	0.3	XX	XX	XX	50,41	9,4	0,0
	3	XX	XX	XX	XX	38,49	8,4
KY 1 (10 μg/ml)	0.3	3,2	0,0	0,0	0,0	0,0	0,0
	3	2,2	2,1	17,16	5,2	0,0	0,0
KY 217 (10 μg/ml)	0.3	2,8	3,3	0,0	0,0	0,0	0,0
	3	X,X	X,X	6,0	5,0	0,0	0,0

In a fourth study, the binding of radiolabeled KY-1 compound to HSV-1 and HSV-2 viral proteins was examined (Example 17). After compound binding, virus proteins were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the gel patterns developed by autoradiography. In Figure 32A, lanes A and B in the figure are autoradiographs of HSV-1 proteins in the presence (lane B) and absence (lane B) of mercaptoethanol, and lanes C and D, analogous patterns for HSV-2 proteins. The lane at the right contains the molecular weight markers, as indicated. The major bands of drug binding in HSV-1 have molecular weights, as determined from SDS-PAGE, of 45, 66, and about 130 kilodaltons. The major bands of drug binding in HSV-2 have similar molecular weights. The major bands which show KY binding in Figure 32B correspond in molecular weight, to HSV glycoproteins gD, gB, and gC.

B. Effect on Non-Enveloped Viruses

The ability of KY compounds to inhibit cell infection by a rhinovirus and adenoviruses 5 and 7 which are non-enveloped viruses, was similarly studied. Vero cells (10^5) were infected with a

rhinovirus in the presence of KY-1, at concentrations ranging between 1-100 μg. Twenty-four hours after virus infection, the cells were examined for cytopathic effect, evidencing viral infection. No reduction in cell clumping was observed at any of the KY drug concentrations tested.

Vero cells were infected with adenovirus in the presence of KY-1, also at concentrations ranging between 1-100 μg, and twenty-four hours after virus infection, the cells were examined for cytopathic effect. No reduction in cell clumping was observed at any of the KY-1 drug concentrations.

In summary, a broad range of macrocyclic compounds are effective inhibitors of cell infection by each of the several enveloped viruses which were studied. Binding studies carried out in particular with respect to HSV viruses indicate that the antiviral activity of the compounds is dependent on binding to virus envelope components, which in turn inhibits virus attachment to infectable cells. The apparent inability of the compounds to inhibit infection of non-enveloped viruses is consistent with this mechanism.

V. Viral Inhibition by Combination of Macrocyclic Compound and an Antiviral Nucleoside Analog

The invention also includes a composition containing a macrocylcic compound of the type described above in combination with a nucleoside analog anti-viral compound. The nucleoside analog compound is one effective to inhibit viral replication at the level of viral replication or transcription. Among the nucleoside analog compounds which are useful in combination with a macrocyclic compound, in accordance with the invention are:

(1) Pyrophosphate analoges, such as phosphoformic acid (PFA), phosphonoacetic acid (PAA), methanediphosphonic acid (MDP), carbonyldiphosphonic acid (COMDP), phosphonoglyoxalic acid (COPAA), and various halogen- and/or methyl-substituted derivatives thereof, which are inhibitors of viral nucleic acid polymerases. In particular, these compounds are known to inhibit herpes virus (Blackburn, Sidwell) and Influenza (Sidwell) infections, and reverse transcriptase activity in retroviruses, such as human HIV.

(2) Base-modified analogs, such as IUDR, trifluorothymidine, AraA, and azidothymidine (AZT), didieoxyinosine (DDI), D4T, dideoxycytidine (DDC), and ribavirin. Trifluorothymidine, IUDR, and AraA are active mainly against herpes virues (Nicolson, 1984a, 1984b). Ribavirin is active against several RNA and DNA viruses (Sidwell), and AZT is active against HIV (Fischl), as are other dideoxynucleoside analogs, such as DDI.

(3) Sugar-modified analogs, such as N-acyl derivatives of 5'-amino-2',5'-dideoxy 5'-ioduridine, sulphonamide derivatives of 5'-amino-5'-deoxythymidine, 2'-deoxy-5-ethyluridine, and N-acyl derivatives, 5'-Sulfate and 5'-sulfamate nucleoside analogs, such as nucleocidin, adenosine 5' sulfamate, and ribavarin, which may act primarily at the level of protein synthesis inhibition (Martin).

(4) Phosphate analogs, including acyclonucleoside phosphonates, such as acyclovir and gangiclovir, and their isosteric phosphonate analogs. These compounds can act as virus-selective substrates for viral thymidine kinases, in the synthesis of nucleoside triphosphate analogs intracellularly (Galbraith). Subsequently, the nucleoside

triphosphate analogs can act as selective substrates for viral DNA polymerase, acting as a chain terminator since the analog does not have the bifunctionality necessary for chain extension (Allen). These compounds have demonstrated anti-viral activity against herpes viruses (Collins), including HSV-1, HSV-2, varicella zoster (VZV), and cytomegalovirus (CMV) (Smith).

Also included in this class are phosphonomethyl ethers of nucleosides, and their acyclic analogs, such as N-(3-hydroxy-2-phosphonylmethoxypropyl)-(HPMP-) and N-(2-phosphonylmethoxyethyl-)(PME-) derivatives of heterocyclic bases. These compounds act specifically against herpes viruses, adenoviruses, cytomegalovirus (DeClercq), poxviruses, vaccinia viruses, and retroviruses.

The ability of the two-compound composition to inhibit viral infection by an enveloped virus is demonstrated in the study reported in Example 18, which examines the viral yields after infection of Vero cells with serial dilutions of HSV-1 or HSV-2 particles, as described above.

Figure 34A shows the drop in HSV-1 viral yields when infected cells are exposed to increasing concentrations of the macrocyclic compound Y-1 alone (solid circles), increasing concentrations of acyclovir alone (open circles), increasing concentrations of acyclovir plus 25 μg/ml Y-1 (solid rectangles), and increasing concentrations of acyclovir plus 50 μg/ml Y-1 (solid ovals). With either drug alone, a maximum decrease in viral yield was slightly less than three logs (orders of magnitude).

The effect of combined compounds was tested at two Y-1 concentrations. At the lower Y-1

concentration of 25 μg/ml, the two compounds together gave over seven logs inhibition in viral yield, i.e., more than tenfold greater than the sum of the inhibition produced by the two drugs alone. At the higher Y-1 concentration, the combined inhibitory effect of the two compounds was several orders of magnitude greater than than the sum of effect of macrocyclic compound and acyclovir alone. Similar results were observed for inhibition of HSV-2 viral yields with combined compound administration, as seen in Figure 34B.

The two compounds are formulated, preferably in a jelly form, at a preferred weight ratio of between about 10:1 to 1:1 macrocyclic compound to nucleoside analog, respectively. The viral-yield plots in Figures 34A and 34B show that a significantly higher level of inhibition was observed when the co-administered compounds were at a ratio of about 5:1 macrocyclic compound to nucleoside. The macrocyclic compound in the composition is preferably selected for optimal activity against the target virus, e.g., a herpes virus, respiratory syncytial virus, or retrovirus, as detailed above. Similarly, the preferred nucleoside analog compound is selected for activity against the target virus (Martin)

One advantage of the combined-drug composition is that substantially lower doses of both types of compounds are required for achieving a selected viral inhibition level, reducing drug side effects in a composition that also is characterized by greater anti-viral activity.

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This section examines the specificity of the viral-inhibition method to enveloped viruses. The

studies reported in subsection A show that the macrocyclic compounds used in the method act, at least in part, by binding selectively to viral envelope proteins, and that this binding blocks virus attachment to infectable cells, thereby inhibiting virus infectivity. These studies are detailed in parent U.S. patent application Serial No. 647,720, filed January 29, 1991. Subsection B examines the inhibitory effect of the macrocyclic on non-enveloped viruses.

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In a second study, the effect of compound when administered prior to, during, or after cell infection by HSV-1 was examined as described in Example 15. In these studies, cells were exposed to one of a series of increasing KY-2 concentrations, and the extent of infection was measured by number of plaques observed 24 hours after infection. The

reduction in plaque formation, expressed as a percent of control, is shown in Figure 31 for cells treated with drug prior to (solid rectangles), during (closed circles), and after (open rectangles). Virus inhibition was seen most significantly when the cells were treated with drug during exposure to virus, indicating that virus inhibition occurs at the period of virus binding to and entry into infectable cells.

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KY 217 (10 μg/ml)	0.3	2,8	3,3	0,0	0,0	0,0	0,0
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In summary, a broad range of macrocyclic compounds are effective inhibitors of cell infection by each of the several enveloped viruses which were studied. Binding studies carried out in particular with respect to HSV viruses indicate that the anti-viral activity of the compounds is dependent on binding to virus envelope components, which in turn inhibits virus attachment to infectable cells. The apparent inability of the compounds to inhibit infection of non-enveloped viruses is consistent with this mechanism.

V. Viral Inhibition by a Composition Containing a Macrocyclic Compound and a Nucleoside Analog Compound

The invention also includes a composition containing a macrocylcic compound of the type described above in combination with a nucleoside analog anti-viral compound. The nucleoside analog compound is one effective to inhibit viral replication at the level of viral replication or transcription. Among the nucleoside analog compounds

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nucleoside triphosphate analogs intracellularly (Galbraith). Subsequently, the nucleoside triphosphate analogs can act as selective substrates for viral DNA polymerase, acting as a chain terminator since the analog does not have the bifunctionality necessary for chain extension (Allen). These compounds have demonstrated anti-viral activity against herpes viruses (Collins), including HSV-1, HSV-2, varicella zoster (VZV), and cytomegalovirus (CMV) (Smith).

Also included in this class are phosphonomethyl ethers of nucleosides, and their acyclic analogs, such as N-(3-hydroxy-2-phosphonylmethoxypropyl)-(HPMP-) and N-(2-phosphonylmethoxyethyl-)(PME-) derivatives of heterocyclic bases. These compounds act specifically against herpes viruses, adenoviruses, cytomegalovirus (DeClercq), poxviruses, vaccinia viruses, and retroviruses.

The ability of the two-compound composition to inhibit viral infection in enveloped virus is demonstrated in the study reported in Example 18, which examines the viral yields after infection of Vero cells with serial dilutions of HSV-1 or HSV-2 particles, as described above.

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The effect of combined compounds was tested at two Y-1 concentrations. At the lower Y-1 concentration of 25 μg/ml, the two compounds together gave over seven logs inhibition in viral yield, i.e., more than tenfold greater than the sum of the inhibition produced by the two drugs alone. At the higher Y-1 concentration, the combined inhibitory effect of the two compounds was several orders of magnitude greater than than the sum of effect of macrocyclic compound and acyclovir alone. Similar results were observed for inhibition of HSV-2 viral yields with combined compound treatment, as seen in Figure 34B.

The two compounds are formulated (e.g., in tablet, jelly, ointment, or injectable form) at a preferred weight ratio of between about 10:1 to 1:1 macrocyclic compound and nucleoside analog, respectively. The viral-yield plots in Figures 34A and 34B a significantly higher level of inhibition was observed when the co-administered compounds were at a ratio of about 5:1 macrocyclic compound to nucleoside. The macrocyclic compound in the composition is preferably selected for optimal activity against the target virus, e.g., a herpes virus, respiratory syncytial virus, or retrovirus, as detailed above. Similarly, the preferred nucleoside analog compound is selected for activity against the target virus (Martin).

One advantage of the combined-drug composition is that substantially lower doses of both types of compounds are required for achieving a selected viral inhibition level, reducing drug side effects in a composition that also is characterized by greater anti-viral activity.

VI. Use of the Composition in Treatment of Viral Infection

In accordance with the invention, calix(n)arene compounds of the type described herein are administered to the site of infection in an individual infected with an enveloped virus, for treatment of the invention. The composition of the invention includes novel includes the calix(n)arene compound contained in a pharmaceutical carrier which is suitable for oral, topical or parenteral administration of the compound. The composition may contain the calix(n)arene compound alone, or in combination with an anti-viral nucleoside analog.

The dosage form of the composition is one which is pharmaceutically effective, i.e., effective to inhibit viral infection of host cells. As seen above, compound doses in the range 1-50 μg/ml are generally effective in inhibiting viral infection of cells. Thus, for many applications, an effective dose is preferably one which produces a concentration of compound in this range at the site of infection. For topical administration, a composition containing between 1-5% or more calix(n)arene is suitable.

In a composition containing both calix(n)arene and nucleoside analog compounds, the composition dose may be substantially lower in one or both compounds, as discussed in the section above.

One of the considerations in the administering the composition, particularly when the drug is administered parenterally or orally, is systemic side effects. Studies conducted in support of the present invention indicate that the calix(n)arene compound, particularly the sulfonic acid compound, may show anti-coagulant activity after oral and intravenous administration. One conclusion from these studies is

that the anticoagulant effect of calix(n)arene in the bloodstream can be effectively blocked by administering a polycationic compound, such as protamine sulfate, by intravenous administration. The protamine administration is timed to correspond to highest blood levels of the calix(n)arene compounds. In a typical method, a dose of protamine equivalent to about 1 mg per 100 heparin anticoagulant units is administered intravenously simultaneously with IV administration of the calix(n)arene, or 1-2 hours after oral administration of the macrocyclic drug. It is generally recommended that protamine be infused slowly (i.e., not more than a total of 50 mg/10 minutes).

Therefore in the case of simultaneous administration of calix(n)arene compound, the rate of co-infusion of the two compounds would be adjusted such that the protamine sulfate was not introduced to the subject at a rate exceeding 50 mg/10 minutes. The composition of the invention can therefore include protamine in an amount effective to reduce the anti-coagulant effect of the macrocyclic compound, when the compound is administered for uptake into the bloodstream. Where the composition also contains a nucleoside analog drug, and lower amounts of macrocyclic drug, the protamine may be reduced or eliminated, due to the lower amounts of macrocyclic compound.

A. Injectable Composition

Studies on the pharmacokinetics and efficacy of intravenously administered composition has been studied. Briefly, it was shown that a macrocyclic compound of the type used in the method, when administered intravenously, (a) is cleared relatively

slowly from the bloodstream ($t_{1/2}$= approx. 5-8 hours), (b) is present predominantly in free form, and (c) retains activity in the bloodstream for inhibiting viral (e.g., HSV-1, HSV-2, RSV, and HIV) infection.

The injectable composition contains the calix(n)arene in a suitable IV solution, such as sterile physiological salt solution. The solution may additionally contain nucleoside analog compound and/or protamine.

B. Topical Composition: Treatment of Genital Herpes Lesions

For inhibiting viral infection of skin and mucosal membrane, the composition is preferably formulated in an ointment form. The use of a topical composition for treatment of genital herpes lesions is illustrated in the following study, which is detailed in Example 13. Briefly, female guinea pigs were infected intravaginally with HSV-2, then treated topically three times daily beginning 6 hours or 48 hours after inoculation with HSV-2, as described in Example 13. Animal groups included control animals (no treatment following virus inoculation), placebo (vehicle treatment), KY-1 in vehicle, or acyclovir. Swabs of vaginal secretion were obtained and assayed for viral activity by a standard CPE assay. The severity of genital lesions was scored on a 0-5+ scale through the period of primary infection (21 days).

Three to four days after HSV-2 inoculation, vesicular lesions appeared on the external genital skin. Lesions progressed to an ulcerative stage by days 7-8 and gradually healed by days 15-21. The effect of topical treatment with the KY-1 preparations on lesion development and severity is

shown in Table 10. The group treated with placebo at +6h had a significantly increased lesion score-day AUC ($P < 0.05$); however, mean peak lesion scores were not different when compared to the untreated control group. Lesion development as determined by both AUC values and mean peak lesion scores was significantly reduced by treatment with 5% KY-1 when given at 6h after infection compared to the placebo ($P < 0.001$). Treatment with 1% KY-1 significantly reduced the AUC at +6h ($P < 0.01$) but not mean peak lesion scores.

Table 10

Treatment	Lesion Score: Area Under Curve	P-Value	Mean Peak Lesion Score	P-Value
Control	37.0	---	3.6	---
Placebo +6h	47.0	<0.05	3.9	NS
Placebo +48h	42.8	NS	3.6	NS
KY 5% +6hr	3.8	<0.001	0.8	<0.001
KY 5% +48h	45.7	NS	3.7	NS
KY 1% +6h	30.8	<0.01	2.9	NS
KY 1% +48h	46.6	NS	4.3	NS
ACV 5% +6h	2.7	<0.001	0.6	<0.001
ACV 5% +48h	45.8	NS	3.8	NS

No sign of any skin irritation from any of the formulations was observed. Throughout the treatment period, the genital skin remained normal in appearance; no redness or swelling was observed. The guinea pigs also remained normal and healthy in appearance throughout the entire study.

In another study using the guinea pig genital model described above, animals were infected with HSV-2, then treated with KY-1 or Y-1 topically at concentrations of 2% or 5% drug. Treatment of animals was initiated 6 or 24 hours post infection, as described in Example 13. Animals were treated and scored daily for severity of infection for 19 days.

The effects of topical treatment with KY-1 and Y-1 on infection are tabulated in Table 11 and compared to treatment with 5% acyclovir (ACV).

Treatment with placebo (vehicle only) resulted in significantly worse infection scores than no treatment in this study. Drug treatment with 2% or 6% Y-1, administered 6 hours post infection, resulted in reduced numbers of animals exhibiting lesions, decreased mean lesion scores and decreased peak lesion score, in comparison to placebo treatment. Likewise, treatment with a 6% formulation of either KY-1 or Y-1 or a 2% formulation of KY-1, administered 24 hours post-infection, resulted in reduced numbers of lesion bearing animals and reduced severity of lesions.

Table 11

Effect of Topical KY-1 and Y-1 on HSV-2 Genital Lesions

Treatment	Time	N	% Animals with Lesions	Lesion Score (AUC)	Mean Peak
None	-	9	66.7	9.1	1.3
Placebo	6 h	10	100	29.0	3.0
Y-1 (2%)	6 h	9	66.7	16.9	1.8
Y-1 (6%)	6 h	10	30	8.1	1.2
Placebo	24 h	10	100	22.3	2.7
Y-1 (2%)	24 h	10	60	22.0	2.3
Y-1 (6%)	24 h	10	40	14.7	1.8
KY-1 (2%)	24 h	10	70	16.2	2.2
KY-1 (6%)	24 h	10	50	17.5	1.7
ACV (5%)	24 h	8	37.5	11.7	1.5

C. Topical Composition: Treatment of Eye Infections

In another embodiment, the topical composition includes a calix(n)arene compound in a ointment or solution form suitable for administering the compound to the eye, e.g., to the corneal surfaces of the eye. The composition may also include a nucleoside compound effective against the target viral infection.

In one treatment method, described in Example 10, graded topical doses of compound Y-1 were administered to the corneal regions of rabbits previously infected with HSV-1. Clinical slit lamp biomicroscopy was used to assess disease severity as measured by average epithelial disease involvement (Figure 33A), conjunctivitis rating (Figure 33B), iritis rating (Figure 33C), and stromal disease (Figure 33D).

By day 7 post infection, the severity of the epithelial disease had peaked, as observed in placebo treated animals (Figure 33A). Conjunctivitis, iritis and stromal disease parameters also progressed throughout the study (Figures 33B-D).

In all four assessments of ocular infection, drug treatment resulted in less severe infection as compared to placebo treatment. All concentrations of Y-1 were effective in reducing the development of HSV-1 induced ocular disease. Therapy with all concentrations of Y-1 were statistically different from each other.

A topical concentration of 12.5 μg/50 μl was the most effective ocular therapy. The epithelial disease scores decreased through day 6 post infection, and rebounded slightly on day 7 post infection. Compared to the other two Y-1 therapies,

this concentration was effective in reducing the development of HSV-1 disease in the eye and was associated with only mild conjunctival, iris, and stromal disease development parameters. A higher concentration (18.75 μg/50 μl) was also effective in reducing the development of corneal epithelial HSV-1-induced disease. However, this concentration of Y-1 appeared to be somewhat toxic to the corneal epithelial surface and to the conjunctiva, iris and stroma. This toxicity was evidenced as an increase in all disease parameters on days 6 and 7 post infection.

Viral titers were recovered from tear film at days 0, 3, 5, and 7 post-inoculation and from epithelial scrapings performed on day 7 post-infection (sacrifice). Viral titers were determined by plaque reduction and multiple regression analysis, as described in Example 10. In the tear film study, a marked reduction of viral titer was observed in all animals given topical doses of Y-1, and this reduction appeared dose-dependent, although no difference was seen at the highest doses (12.5 and 18.75 μg/50 μl). A dose-dependent reduction in viral titer was observed in the scrapings taken on day 7.

Based upon these studies, dose efficacy/range was generated. The optimal concentration of compound appeared to be 12.5 μg/50 μl in this study.

D. Oral Composition

Studies conducted in support of the present invention have shown that a macrocyclic drug of the type used in the invention is available in the plasma for a period from about 0.5 hrs. after oral administration (e.g., by gavage), with a peak at about 2-4 hours. The period of effective drug

concentration in the bloodstream is roughly between 4 and 18 hours after IV administration. The relatively short distribution volume halflife of the drug, reflecting distribution to extracorporeal body compartments when the compound is administered intravenously, is generally advantageous in the case where drug is one which shows anti-coagulant side effects, since the concentration of compound in the bloodstream can be more closely titrated.

VII. Topical Composition for Inhibiting Infection by Sexually Transmitted Enveloped Viruses

In a method of the invention, a topical composition containing a macrocyclic compound such as described above is administered to an area or areas of the body where sexual contact is likely to occur. Such areas may include the skin surfaces of the anal-genital regions and the mouth, and also mucous membrane tissues of the vagina, rectum, mouth, and throat. Studies carried out in support of the invention indicate that the macrocyclic compounds described above are compatible with topical administration on skin and mucous membranes; i.e., that the compounds do not give rise to signs of irritation, such as swelling or redness.

The topical composition may include a pharmaceutically acceptable carrier adapted for topical administration. Thus, the composition may take the form of a suspension, solution, ointment, lotion, sexual lubricant, cream, foam, aerosol, spray, suppository, implant, inhalant, tablet, capsule, dry powder, syrup, balm or lozenge, for example. Methods for preparing such compositions are well known in the pharmaceutical industry. In

addition to the macrocyclic compound, the composition may include an antiviral nucleoside analog such as discussed in section V above.

In one preferred embodiment, the composition is a lubricating jelly (sexual lubricant) which includes a lubricating jelly vehicle, and dissolved in the vehicle, a macrocyclic compound of the invention. The jelly vehicle acts in part to minimize abrasion during sexual contact, thereby reducing the likelihood of entry of an enveloped virus into damaged tissue and subsequent entry into the blood stream. As discussed in section IVA, the macrocyclic compounds of the invention are effective to bind tightly to enveloped viruses, thereby inhibiting virus attachment to infectable cells. Thus, the macrocyclic compound in the jelly can intercept enveloped virus particles before infection can occur.

The dosage form of the composition is one which is pharmaceutically effective, i.e., effective to inhibit infection by a sexually transmitted enveloped virus. As seen above, compound doses in the range 1-75 μg/ml are generally effective in inhibiting viral infection of cells. Thus, for many applications, an effective dose is preferably one which produces a concentration of compound in this range at the site of infection. For topical administration, a composition containing between 1-10% of macrocylic compound is suitable. In a composition that contains an antiviral nucleoside analog compound (e.g., section V) in addition to a macrocyclic compound of the invention, the amount of compound in the composition may be substantially lower for one or both compounds.

A. Topical Composition: Prevention of Genital Herpes Infection

The ability of Y-1, as part of a lubricating jelly formulation, to inhibit cytopathic effects in cells exposed to HSV-1 and HSV-2 was assessed in the study detailed in Example 19. Jelly formulations consisting of a commercial lubricating jelly (K-Y Jelly, Johnson & Johnson) and a selected amounts of Y-1 were used. In the study, cells were exposed to HSV-1 or HSV-2 virus and, at the same time, to a Y-1 jelly composition at final drug concentrations of 5-40 μg/ml. Twenty-four hours after inoculation, the cells were inspected for cytopathic effects (i.e., round cell formation).

The results are shown in Table 12. A "+" symbol in the indicates that the concentration of Y-1 was effective in inhibiting cytopathic effects (CPE's). A "-" symbol indicates that CPE was observed.

Table 12

Formulation	Y-1 Conc.	HSV-1 (KOS)	HSV-2 (333)
5% Y-1 in K-Y Jelly	5 ug/ml	-	-
	10 ug/ml	+/-	+/-
	20 ug/ml	+	+
	40 ug/ml	+	+
10% Y-1 in K-Y Jelly	5 ug/ml	-	-
	10 ug/ml	+/-	+/-
	20 ug/ml	+	+
	40 ug/ml	+	+
20% Y-1 in K-Y Jelly	5 ug/ml	-	-
	10 ug/ml	+/-	+/-
	20 ug/ml	+	+
	40 ug/ml	+	+
Y-1 Alone	5 ug/ml	-	-
	10 ug/ml	+/-	+/-
	20 ug/ml	+	+
	40 ug/ml	+	+

The results indicate that the various Y-1-containing jelly compositions were effective to significantly inhibited cell infection at drug concentrations of about 20 μg/ml or greater.

B. Inhibition of HIV Infection

In another study, lubricating jelly formulations containing various amounts of Y-1 were tested for inhibition of cytopathic effects in cells infected with one of two HTLV-III strains, HTLV-III_B and RF-II (Example 20). Briefly, cells chronically infected with HTLV-III_B or RF-II were incubated in the presence of serial dilutions of the selected Y-1-containing jelly compositions, and were then cocultured with indicator cells. The extent of syncytia formation was scored under phase microscopy.

The results, shown in Table 13, were scored as follows: "-" represents absence of cell fusion (or complete blockage of fusion), and 4+ represents a level of cell fusion indistinguishable from untreated controls.

Table 13

Y-1 ug/ml	20% Y-1 in K-Y Jelly	10% Y-1 in K-Y Jelly	5% Y-1 in K-Y Jelly
500	-	-	-
250	-	-	-
125	-	-	-
63	+/-	+/-	+/-
32	4+	4+	4+
16	4+	4+	4+
8	4+	4+	4+
6	4+	4+	4+
4	4+	4+	4+

As can be seen, exposure of the cells to Y-1 concentrations greater than about 63 ug/ml was associated with inhibition of HIV-induced cell fusion. The results indicate that Y-1 formulated in a jelly composition is effective for inhibiting cell infection by the HTLV strains tested.

VIII. Contraceptive Device

In another aspect, the invention includes a physical-barrier type device, in combination with a macrocyclic of the type described above, for use in inhibiting infection by sexually transmitted enveloped viruses. In one embodiment, the device includes a physical barrier-type device (e.g., a contraceptive device), and coating the device, a lubricating composition composed of a lubricating jelly vehicle containing a macrocyclic compound of the invention.

In one embodiment, the device is a condom, where the lubricating composition is coated on the outer surface of the condom. The contraceptive can also be a condom in which the macrocycle-containing composition coats the inner surface of the condom, for the purpose of combining with any semen expressed into the condom. The condom may be of the conventional type used by males (i.e., for placement on a male penis), or may be of an insert-type such as can be worn by a female, where the device includes a condom element that is inserted into the vagina prior to initiation of heterosexual intercourse, for example.

The device may also take the form of a cervical cap, diaphragm, or contraceptive sponge which is placed in the vicinity of the cervix. Accordingly, a cervical cap or diaphragm is coated or infused with a

jelly or cream, for example, which contains a macrocyclic compound of the invention. Similarly, a contraceptive sponge may be imbued with a solution, cream, or the like, which contains a macrocyclic compound of the invention.

In a study to assess the compatibility of the macrocyclic compounds of the invention with latex condoms, a 20% w/w mixture of Y-1 in K-Y jelly was applied to the interior of several brands of latex condoms. A 50 ml conical centrifuge tube was inserted into each condom to simulate an erect penis, and the open end of each condom was sealed with dialysis clips. The sealed condoms were then placed in double-distilled water at 37°C for 24 hours.

After dialysis, the water was analyzed by HPLC for the presence of Y-1. At the sensitivity of the assay (5 ug/ml) no Y-1 was detected for any of the condoms, showing that the macrocyclic compound did not seep through the latex condoms. Thus, the macrocyclic compounds of the invention are compatible with latex contraceptive devices for preventing the transmission of viral infection during sexual intercourse.

The following examples illustrate methods of preparing macrocyclic compounds in accordance with the invention, and the use in inhibiting infection. The examples are intended to illustrate but not limit the scope of the invention.

Materials

All chemical reagents were obtained from Aldrich Chemical Co., or from other commercial sources.

Example 1

Preparation of Naphthalene Macrocyclic Compounds

A. KY-1 (R_1=OH, R_2=SO_3Na, R_3=H, R_4= $>CH_2$)

To a 41 mM aqueous solution (50 ml) of disodium chromotropic acid, 15 ml of 37% formaldehyde was added, giving a final molar ratio of 5:1 formaldehyde:chromotropic acid. The mixture was reacted with stirring in a stoppered flask at room temperature for 1 week. The resulting dark red solution (70 ml) was filtered under vacuum, and the filtrate, after being concentrated was precipitated by adding 200 ml of acetonitrile. The precipitated product was collected by filtration and taken to dryness under vacuum. The yield of KY-1 was 95%. The compound was characterized as follows:
Melting point (M.P.)> 300°C;
HPLC in $CH_3CN/MeOH/H_2O$/TFA: 14'48" single broad peak;

(IR/KBr) = 3425 (OH), 1638 (Ar), 1181, 1044 (SO_3) cm^{-1};
UV (H_2O): 238.0, 358.5 nm
Mol Weight: 1505 (M+1) by mass spectroscopy;
H^1 NMR(CD_3OD), chemical shifts on the γ scale: 5.20 (CH_2, 8.01 (ArH) ppm;
C^{13} NMR (D_2O), chemical shifts on the γ scale: 27.19, 120.18, 121.69, 122-06, 122-67, 133-30, 142.97, 154.42 and 181 ppm. Analysis: $(C_{22}H_{10}O_{16}S_4Na_4)_2 \times 6$ H20 or $(C_{22}H_{11}O_{16}S_4Na_4)_2 \times 5$ H_2O
Found: C 33.17, H 2.54, Na 11.93
Calculated: C 32.75, H 2.23, Na 11.41; C 33.16, H 2.13, Na 11.56.

B. KY-3 (R_1=OH, R_2=SO_2NH_2, R_3=H, R_4= $-CH_2-$)

KY-1 (2mM) was treated with 5 ml chlorosulfonic acid and the mixture was stirred at 50°C for one-half

hour. The resultant mixture was added to 20 g of crushed ice to precipitate the product, which was collected by filtration and then washed with ether.

The crude product was dissolved in 100 ml of 25% ammonium water solution and allowed to react for 2 hours at room temperature. The mixture was concentrated in vacuo and the remaining oil was dissolved in a small amount of water and filtered. The product was precipitated by adding acetonitrile to the filtrate and collected by filtration and washing with acetonitrile. The compound was characterized as follows:

Melting point (M.P.)> 300°C;

Mass spec: 1452 ($M-7NH_2$);.

HPLC in $CH_3CN/MeOH/H_2O/TFA$: 11'46" single peak;

(IR/KBr) = 3430 (OH), 3187, 1686 (NH_2), 1637 (Ar), 1211, 1110, 1044 (SO_3) cm^{-1};

UV (H_2O): 246 nm;

H^1 NMR(D_2O), chemical shifts on the γ scale: 5.15 (CH_2), 7.5-8.2 (ArH) ppm;

Analysis: $(C_{44}H_{40}O_{26}S_{10}N_{12}Na_4)-16H_2O$

Found: C 28.62, H 3.93, N 8.82, S 17.17, Na 5.44;

Calculated: C 28.51, H 3.89, N 9.07, S 17.28, Na 4.97;

C. KY-42 (R_1=OH, R_2=SO_3Na, R_3=H, R_4= >CHCOOH)

Chromotropic acid, disodium (10mM) in 50 ml water was mixed with glyoxylic acid (10.0 mM, in 5 ml water) and 10 ml of 37% hydrogen chloride at room temperature. The mixture was boiled for 8 hours and the color of the solution turned to dark red. The resultant solution was added to 50 ml of water and filtered. The filtrate was concentrated and ethanol was added to precipitate the product of KY-42. The

yield was 87%. The compound was characterized as follows:

Melting point (M.P.)> 300°C;

Mass spec: 1623 ($M-3H_2O$).

HPLC in $CH_3CN/MeOH/H_2O/TFA$: 10'36" single peak;

(IR/KBr) = 3452 (OH), 1801, 1719 (Co), 1638 (Ar), 1206, 1050 (SO_3) cm^{-1};

UV (H_2O): 238.0, 351.5, 520 nm;

H^1 NMR(D_2O), chemical shifts on the γ scale: 7.10 ($C\underline{H}CO_2H$) 8.00 (ArH) ppm;

C^{13} NMR (D_2O), chemical shifts on the γ scale: 116.04, 118.90, 120.94, 121.27, 122.30, 124.30, 124.68, 126.60, 128.37, 136.48, 136.71, 140.50, 143.93, 144.26, 145.75, 152.01, 154.33, 156.01, 156.67;

Analysis: $(C_{48}H_4O_{40}S_8Na_8)_4-4H_2O$

Found: C 32.74, H 2.50;

Calculated: C 32.58, H 2.71;

D. <u>KY-123 (R_1=OH, R_2=SO_2Na, R_3=H, R_4= >CH_2)</u>

KY-1 (2mM) was treated with 5 ml chlorosulfonic acid and the mixture was stirred at 50°C for one-half hour. The resultant mixture was added to 50 g of crushed ice to precipitate the product which was collected by filtration and then washed with ether. The crude sulfonyl chloride product was treated with sodium sulfite (20 mM) in 4 ml water. The reaction mixture was kept slightly alkaline by addition at intervals of small portions of 50% NaOH for 2 days. After solvent removal, ethanol was added to precipitate the product, which was acidified by addition of 50% H_2SO_4, followed by addition of ethanol to precipitate sodium sulfate. The ethanol phase was mixed with ether (1:2, v/v) to precipitate the desired product. Product yield was 39%.

E. KY-147 (R_1=OH, R_2=SO_2NHCH_3, R_3=H, R_4= $>CH_2$)

N-methyl chromotropic acid chloride was formed by reacting chromotropic acid (disodium salt) with sulphonylchloride in the presence of DMF. The reaction was carried out with stirring at 80°C for 4 hours. After removal of solvent and excess of thionylchloride in vacuo, ether was added to precipitate the chromotropic acid chloride which was subsequently collected by filtration and washed with ether. The crude product was added to 20 ml of methylamine and stirred for 2 hours. After removal of all solvent from the resultant substance, the residue was dissolved in a 200 ml of cold methanol and filtered. The filtrate was added with acetonitrile to precipitate the product chromotropic acid methyl sulfonamide. Yield 56%.

The chromotropic acid methyl sulfonamide (2mM) in 3 ml water was reacted with 37% formaldehyde (1ml) at room temperature for one week. Acetonitrile was added to precipitate the product which was collected by filtration and washed by acetonitrile. Yield was 85%.

F. KY-151 (R_1=OCH_3, R_2=SO_3Na, R_3=H, R_4= $>CH_2$)

KY-1 (50mM) was dissolved in 80 ml of NaOH water solution (0.2M NaOH) and heated to 50°C, dimethylsulfate (0.2M) was added slowly for 1 hour. The mixture was continuously stirred for another 2 hours and left at room temperature for 2 days. Saturated NaCl solution (100 ml) was added to the resultant substance and filtered. The precipitate was washed with ethanol, acetonitrile and ether sequentially. The dry substance was dissolved in 100 ml of methanol and filtered. The filtrate was concentrated and

ether was added to precipitate the dimethyl ether of chromotropic acid, disodium.

G. KY-158 (R_1=OH, R_2=SO_2CH_3, R_3=H, R_4= >CH_2)

KY-1 from Example 1A was first treated with thionyl chloride to produce chromotropic acid sulfonyl chloride. This compound was reduced by excess sodium sulfite in the presence of sodium bicarbonate to produce the corresponding sodium sulfonate salt of cyclized chromotropic acid (R_2 = SO_2Na). The sulfonate salt was treated with dimethyl sulfate in the presence of $NaHCO_3$, and worked up as as described in Example 1A. Product yield was about 21%.

H. KY-175 (R_1=OH, R_2=SO_3CH_3, R_3=H, R_4= >CH_2)

Chromotropic acid was first treated with thionyl chloride to produce chromotropic acid sulfonyl chloride. This compound was then treated with sodium methoxide in methanol in the presence of sodium salt. The product was worked up as described in Example 1A to form the macrocyclic compound. Product yield was about 29%.

I. KY-285 (R_1=$OCOCH_3$, R_2=SO_3Na, R_3=H, R_4= >CH_2)

KY-1 from Example 1A (0.66 mmole) was dissolved in 3 ml water containing 0.1 g NaOH. To this was added 1 g acetyl chloride (13 mmole) and the reaction was allowed to proceed at room temperature overnight with stirring. After solvent removal, 25 ml ethanol was added to precipitate the product. The crude product was dissolved in methanol and filtered. The filtrate was allowed to precipitate, giving a 87% yield.

J. KY-346 (R_1=OH, R_2=SO_3Na, R_3=H, R_4= $-CH_2-N(CH_3)CH_2$)

Chromotropic acid disodium salt, was dissolved in 80 ml of water at a concentration of 50 mM with stirring at 50°C until the solution turned to clear, hexamethylenetetramine (50 mM) was then added to above solution with continuous stirring at the same temperature for additional two hours. At this time, the color of this mixture converted to dark blue. The mixture was allowed to stir at room temperature for 2 days. The resultant dark blue solution was filtered and the filtrate was concentrated, evaporated by flask, which was subsequently treated with 200 ml methanol to precipitate the product KY-346. The yield of KY-346 was 85%. The compound was characterized as follows:

M.P.>300°C;

HPLC in $CH_3CN/MeOH/H_2O/TFA$: 13'07" single peak;

(IR/KBr) = 3425 (OH), 1626 (Ar), 1197, 1052 (SO_3) cm^{-1};

UV (H_2O): 232.0, 377.5 nm

Analysis: $(C_{13}H_{11}O_8NS_2Na_2)_4 \times 12\ H_2O$

Found: C 33.17, H 3.13, N 2.75

Calculated: C 33.98, H 3.59, N 2.96.

Molecular weight: 1668 by gel filtration.

Example 2

Preparation of Calix(n)arene Compounds

A. Y-49 (R_1=OH, R_2=SO_3H, R_6=$-CH_2-$, n=4)

4-tert-butylcalix(4)arene (10 g) was treated with 200 ml of concentrated H_2SO_4 at room temperature for 0.5 hour and then at 75-85°C oil bath for another 4 hours. The reaction was completed when no water-insoluble material was detected. The resultant oil was dropped into 500 g of crushed ice and the solution was filtered by reduced pressure. After the

water removed away from the filtrate, acetonitrile (500 ml) was added to the residual and allowed to stand for 4 hours to precipitate the crude product which was then collected by filtration and washed with acetonitrile, ethyl acetate and ether. Yield 8 g (73%). The pure product was furnished by recrystallization of the crude compound with methanol-ether or methanol-acetonitrile system. The single crystal compound was also found in the recrystallization process.

Similar methods were used in the synthesis of Y-77 (R_1=OH, R_3=SO_3H, R_4= $-CH_2-$, n=6) and Y-1 (R_1=OH, R_3=SO_3H, R_4=$-CH_2-$, n=8).

D. KY-225 (R_1= -OH, =O), R_2=SO_3H, R_4= >CH_2, > CH, n=4)

4-tert-Butylcalix(4)arene (1 g) was treated with 10 ml of 95-98% sulfuric acid at room temperature for 0.5 hours then at 160°C for 5 minutes. After the resultant mixture was cool, the mixture was poured slowly into 100 ml of crushed ice and filtrated. The solution was evaporated and the residual was added with 300 ml acetonitrile to produce great amount of precipitate which was collected by filtration and washed with acetonitrile. The crude product was dissolved in 20 ml methanol and the product was precipitated by addition of diethyl ether. Yield was 84%.

Similar methods were used in the synthesis of Y-48 (R_1= -OH or =O, R_3=SO_2H, R_4= $-CH_2-$, n=6) and Y-226 (R_1= -OH or =O, R_2=SO_3H, R_4= $-CH_2-$, n=8).

C. O-Acetylate of Y-1 (R_1= -$OCOCH_3$, R_2=SO_3H, R_4= >CH_2, n=8)

Y-1 (0.75 g) was stirred in dry acetic anhydride (30 ml) overnight. The reaction was continued until the material was dissolved in the solvent. After cooling to room temperature, the suspension was filtered. The solid was washed twice with acetonitrile and dried in vacuo. The material was washed and recrystallized.

^{13}CNMR (D20, δ): 173.9, 151.6, 144.1, 135.6, 130.1, 34.2, and 22.4.

D. Y-78 (R_1= -OH, R_2=SO_2NH_2, R_4= >CH_2, n=8)

Under nitrogen, Y-1 (1 g) is heated at 60-70°C with chlorosulfonic acid (20 ml) for 1 hour. After cooling to room temperature, the oily material is poured into ice water, and the precipitate is filtered. After washing the precipitate with cold water, the material is added to 50 ml of solution containing 5.7 g glycine and 2.1 g NaOH, and stirred for 2 hours at room temperature. The crude product was dissolved in 100 ml of 25% ammonium water solution and allowed to react for 2 hours at room temperature. The mixture is concentrated in vacuo and the remaining oil is dissolved in a small amount of water and filtered. The product is precipitated by adding acetonitrile to the filtrate and collected by filtration and washing with acetonitrile.

E. Glycyl sulfonamide of Y-1 (R_1= -OH, R_2=$SO_2NHCH_2CO_2H$, R_4= >CH_2, n=8)

Under nitrogen, Y-1 (1 g) is heated at 60-70°C with chlorosulfonic acid (20 ml) for 1 hour. After cooling to room temperature, the oily material is poured into ice water, and the precipitate is

filtered. After washing the precipitate with cold water, the material is added to 50 ml of solution containing 5.7 g glycine and 2.1 g NaOH, and stirred for 2 hours at room temperature. After removal of all solvent from the resultant substance, the residue is dissolved in a 200 ml of cold methanol and filtered. The filtrate is added with acetonitrile to precipitate the product.

F. Acetyl-Bridged Y-49 (R_1= -OH, R_2=SO_3H, R_4= -$CHCO_2H$-, n=4)

4.3 g of p-hydroxybenzenesulfonic acid was treated with g gram of glyoxylic acid in 30 ml 18% conc. HCl for 2 hours at 100°C. After the reaction product was dried under reduced pressure, 50 ml of methanol was added and insoluble impurities were removed by filtration. The product was precipitated from the filtrate by addition of ether then collected by filtration and dried in vacuo.

G. Toluene Sulfonyl Ester of Y-49 (R_1= -$SO_3C_6H_4CH_3$, R_2=SO_3H, R_4= >$CHCO_2H$, n=4)

Under nitrogen is added toluenesulfonyl chloride (1.9 g) to a suspension of dry sodium carbonate (1.06 g), dry dimethylformamide (10 ml) and Y-49 (0.75 g). After an overnight reflux, the resulting mixture is cooled to room temperature and filtered. The filtrate is diluted with ether to precipitate out the crude product. Recrystallization from acetonitrile/ether solvent provided the product.

H. Carboxylic Acid Derivative of Y-49 (R_1= -CO_2H, R_2=SO_3H, R_4= >$CHCO_2H$, n=4)

Under nitrogen, trifluoromethanesulfonic anhydride (1.0 ml) is added to ice cold dry

dichloromethane solution (10 ml) of 2,6, di-tert-butyl-4-methylpyridine (1.25 g) and 4-tert-butylcalix[4]arene (0.65 g). After overnight stirring at room temperature, the mixture is diluted with pentane (10 ml) and filtered. The filtrate is extracted with ice cold 1N aqueous NaOH solution, ice cold 1N aqueous HCl solution, then saturated aqueous NaCl solution, dried over anhydrous sodium sulfate, filtered through a pad of silica gel and concentrated in vacuo. The residue is dissolved in a mixture of dry diisapropylethylamine (10 ml), trimethylsilyl cyanide (0.5 ml) and palladium tetrakis-triphenylphosphine (20 mg). After an overnight reflux under nitrogen and then cooling to room temperature, ether (50 ml) was added and the resulting suspension was filtered. After concentration of the filtrate in vacuo and silica gel chromatography (hexane/ethyl acetate eluent), the cyano intermediate is heated at 80°C with concentrated sulfuric acid (10 ml) for 3 hours, diluted with water (10 ml) and refluxed overnight. After cooling to room temperature, the resulting mix is added to charcoal (0.5 g) and ice (50 g). After filtration, the resulting filtrate is concentrated in vacuo to ca 15 ml in volume and the resulting solid was filtered. The solid is dissolved in a minimal amount of methanol and precipitated out by adding ether. Final purification by reverse phase C18 chromatography (methanol/water eluent) provide the product.

I. Methyl Ether of Y-1 (R_1 =OMe, R_2=SO_3Na, R_4 >CH_2, n = 8)

A mixture of Y-1 (447 mg), NaOH (6 N in water, 1.53 ml), and dimethylsulfate (9 ml) was heated at

60°C for 20 hours. The resulting mix was added dropwise into stirring absolute ethanol (100 ml). The resulting suspension was centrifuged (9000 rpm, 20 min) and then the supernatant was removed. Twice, the resulting solid was dissolved in water (6 ml), and the resulting solution was treated as above with ethanol, centrifuged, and supernatant removed. The remaining solid was lyophilized to yield the product (420 mg).

^{13}CNMR (D20, δ): 161.2, 140.9, 137.6, 129.5, 63.6, and 33.5.

J. XXVI. (R_1= -OH, R_2= H, R_3=R_5= H, R_4= -CH_2-, n=4)

Calix(4)arene XXVI is prepared from 4-tert-butylcalix(4)arene (XXV; Fig. 13) as described (Gutsche, Levine, and Sujeeth, 1985). A hot solution of 5.0 g (6.75 mmol) of XXV in 250 ml of toluene is placed in a 500 ml three-necked round-bottom flask fitted with a mechanical stirrer and a gas inlet tube. The solution is cooled to 50-55°C, treated with 5.0 g (37 mmol) of anhydrous $AlCl_3$, and stirred for 2 h at 50-55°C in an inert atmosphere. The mixture is cooled in an ice bath and stirred with 125 ml of 1 N HCl for 30 min, and the organic phase is separated and washed, dried, and evaporated to leave a yellow residue. This is triturated with 500 ml of ether, and the insoluble material is recrystallized from $CHCl_3$-CH_3OH to yield 1.9 g (66%) of XXVI as off-white microcrystals. m.p. 313-318°C.

K. XXVIII. (R_1= -OH, R_2= COOH, R_3=R_5= H, R_4= -CH_2-, n=4)

Calix(4)arene XXVIII is prepared as described (Yilmaz and Vural). Known p-acetyl-calix(4)arene

(XXVII; 1.3 g) (Yilmaz and Vural, 1991; No et al., 1986) is dissolved in 50 ml of 2 N aqueous NaOH. A solution of iodine (8 g) and potassium iodide (20 g) in 40 ml of water is added and the mixture stirred. The solution is warmed on a water bath for 1 h. Iodoform is removed by filtration, and $NaHSO_3$ (20 g) is added to the filtrate. Concentrated HCl is then added to the filtrate to produce a pale yellow precipitate which is then filtered off, washed with water, and dried. The crude product is dissolved in 10% aq. $NaHSO_3$ and treated with charcoal. After filtration, the solution is acidified with 1 N HCl. The precipitated product is collected by filtration, washed with distilled water until free of Cl^-, and dried in a vacuum desiccator, yielding 1.04 g (79%) of XXVIII. m.p. 320°C (dec.).

L. XXXI. (R_1= -OH, R_2= $-CH_2COOH$, R_3=R_5= H, R_4= $-CH_2-$, n=4)

Calix(4)arene derivative XXXI is prepared via p-(dimethylamino)methyl-calix(4)arene (XXIX) as described (Gutsche and Nam, 1989).

To a solution of 15.9 g (39.5 mmol) of calix(4)arene (XXVI) in 360 ml of THF are added 45 ml of acetic acid, 22.5 g (0.2 mol) of 40% aqueous dimethylamine, and 16.2 g (0.2 mol) of 37% aqueous formaldehyde. The reaction mixture is stirred for 24 h at room temperature, the solvents are removed under vacuum, and the residue is dissolved in 250 ml of water. The aqueous solution is extracted twice with 200 ml of ether and neutralized with 10% K_2CO_3 solution, and the precipitate that forms is removed by suction filtration. The product is dried under vacuum and then recrystallized from chloroform to

give 19.1 g (78%) of p-(dimethylamino)methyl-calix(4)arene XXIX as white needles.

To a solution containing 16.3 g of p-(dimethylamino)methyl-calix(4)arene in 220 ml of DMSO is slowly added 9.57 ml (0.15 mol) of methyl iodide. After the reaction mixture is stirred for 30 min at room temperature, 15 g (0.3 mol) of NaCN is added, and the mixture is heated for 2 h at 80°C under a nitrogen atmosphere. The solution is then cooled, treated with 1 liter of ice water, acidified with 2 N HCl, filtered, and air-dried. The crude product is recrystallized from CH_3CN to yield 12.8 g (88%) of p-cyanomethyl-calix(4)arene XXX as a pale yellow solid.

p-Cyanomethyl-calix(4)arene (0.5 mmol) is then added to a solution of DMSO (25 ml) and conc. aqueous HCl(5 ml) and refluxed overnight. After dilution with water (100 ml) at room temperature, the precipitate is collected by filtration and recrystalized from methanol to provide purified XXXI.

M. XXXIII. (R_1= -OH, R_2= $-CH_2CH_2COOH$, R_3=R_5= H, R_4= $-CH_2-$, n=4)

Calix(4)arene derivative XXXIII is prepared as described (Gutsche and Nam, 1989).

To a solution of 3.26 g (5 mmol) of (dimethylamino)methyl-calix(4)arene (XXIX; Example L) in 80 ml of DMSO, 1.90 ml (30 mmol) of methyl iodide is added. After the mixture is stirred for 30 min, sodium diethyl malonate, prepared from 1.20 g of Na, 7.28 g of diethyl malonate, and 28 ml of EtOH, is added, and the reaction mixture is heated for 2 h at 80°C in an atmosphere of nitrogen. The solution is then cooled, poured onto 200 ml of ice-water, acidified with 2 N HCl, and worked up in the usual fashion to give 5.50 g (99%) of p-

(diethylmalonyl)methyl-calix(4)arene XXXII as crude product. Hydrolysis and decarboxylation is effected by dissolving the crude product in 100 ml of DMSO and 30 ml of conc. HCl and heating at 120°C for 10 h in an atmosphere of nitrogen. The mixture is then cooled, poured onto 500 ml of ice-water, stirred for 10 min, and filtered. The precipitate is recrystallized from acetone-ethyl acetate to give 2.42 g (69%) of XXXIII as colorless crystals. m.p. 224.

N. XXXVI (R_1= -OH, R_2= $-PO_3H$, R_3=R_5= H, R_4= $-CH_2-$, n=4)

Derivative XXXVI is prepared by adaptation of the methods of Arduini et al. and Hirao et al.

Calix(4)arene (XXVI) is refluxed with $Hg(OCOCF_3)_2$ in $CHCl_3$, giving an almost quantitative yield of the tetra-(Hg-$OCOCF_3$) calixarene derivative. Following evaporation of the $CHCl_3$, metal iodine exchange is carried out by reaction of the calixarene derivative with I_2 in $CHCl_3$, giving p-iodo-calix(4)arene XXXIV as a brown compound in 40% yield.

A concentrated toluene solution of $HPO(OEt)_2$ (10 mmol), triethylamine (10 mmol), $Pd(PPh_3)_4$ (0.3 mmol) and p-iodo-calix(4)arene (1.0 mmol) is stirred at 100°C under nitrogen atmosphere for 3 days. After dilution with ether (50 ml) at room temperature, the reaction mixture is filtered and then concentrated under high vacuum (100°C, at < 0.1 mm Hg). The resultant concentrate is purified by silica chromatography to obtain purified phosphonate diester (XXXV), which is then refluxed overnight in 6 N HCl (5 ml) to produce the phosphonic acid product. After removal of solvent (100°C, at < 0.1 mm Hg), the solid

is recrystallized from methanol to yield purified XXXVI.

O. XXXIX. (R_1= -OH, R_2= $-CH_2PO_3H$, R_3=R_5= H, R_4= $-CH_2-$, n=4)

Calix(4)arene derivative XLIV is prepared via p-chloromethyl-calix(4)arene as described (Almi et al.).

To a solution of 1.0 g (2.4 mmol) of calix(4)arene (XXVI) and 14.4 g (81 mmol) of chloromethyl-n-octyl ether in 100 ml of $CHCl_3$ cooled at -10°C, is added 4.7 ml (40.3 mmol) of $SnCl_4$ dropwise over about 15 min. The cooling bath is then removed, and the reaction mixture is kept at room temperature until all of the calixarene starting material has reacted (after ~50 min), as judged by thin layer chromatography (hexane:ethyl acetate = 4:3). Water is then added slowly and the two phases are allowed to separate. The organic layer is washed twice with distilled water and is then dried over Na_2SO_4. Following removal of the Na_2SO_4, the solvent is evaporated to give a residue that is then washed with n-hexane and filtered, giving 1.23 g (80%) of product, p-chloromethyl-calix(4)arene XXXVII.

Derivative XXXVII (1 g, 1.6 mmol) is refluxed for 6 h in 20 ml of triethyl phosphite. Excess triethyl phosphite is then removed by distillation, and the resultant solid residue (phosphonate diester XXXVIII) is dried under vacuum for 8 h. A solution of 20% HCl (60 ml) is added and the resultant reaction mixture is refluxed for 20 h. The solvent is then removed by evaporation, and the resultant precipitate is filtered, washed first with methanol and then with $CHCl_3$, and dried under vacuum to give

1.11 g (80%) of XXXIX as a white solid. m.p. 360°C.

P. XLIV. (R_1= -OTs, R_2= $-CH_2CH_2Br$, R_3=R_5= H, R_4= $-CH_2-$, n=4)

p-2-Bromoethyl-calix(4)arene derivative XLIV is prepared as described (Gutsche, Levine, and Sujeeth, 1985; Gutsche, Dhawan, et al., 1983).

(a) To a solution of calix(4)arene (XXVI; 2.14 g) in 100 ml of THF and 10 ml of DMF is added 2.0 g of NaH followed by 28 g of allyl bromide. The mixture is refluxed 1 h, after which the THF is removed by evapcration, and the residue is partitioned between water and $CHCl_3$. The $CHCl_3$ extract is washed with water, dried, and evaporated, and the residue is recrystallized from 95% ethanol to give 2.18 g (74 %) of O-allyl-calix(4)arene XL as colorless needles.

(b) A solution of 1.66 g (2.84 mmol) of the O-allyl calix(4)arene in 25 ml of N,N-diethylaniline is heated at reflux for 2 h in an inert atmosphere. The solution is cooled, poured into 250 ml of ice-water, stirred with 250 ml of concentrated HCl, and filtered to yield a crude product, which is then crystallized from isopropanol to afford 1.22 g (74%) of p-allyl-calix(4)arene XLI as off-white needles, m.p. 245-248°C.

(c) A solution of 2.09 g (3.57 mmol) of p-allyl-calix(4)arene in 100 ml of dry THF is treated with 1.0 g (42 mmol) of NaH followed by 4.0 g (21 mmol) of p-toluenesulfonyl chloride, and the mixture is heated at reflux for 1.5 h. The solvent is removed by evaporation to leave a light brown oil, which is dissolved in 100 mL of $CHCl_3$, cooled in an ice bath, and treated with 100 ml of ice-water. The

organic phase is dried and evaporated, and the residue is recrystallized from isopropanol to yield 3.41 g (79.5%) of tosylated p-allyl-calix(4)arene (XLII).

(d) A solution of 3.50 g of tosylated p-allyl-calix(4)arene in 60 ml of CH_2Cl_2 and 40 mL of CH_3OH is cooled in a dry ice-acetone bath and treated with ozone until it retains a blue color (10-15 min). Nitrogen is bubbled through the solution until the blue color disappears, and 2 g of $NaBH_4$ is added. The solution is stirred at room temperature for 3-4 h, poured into ice cold, dilute HCl solution, and worked up in conventiaonal fashion to yield a crude product as a white resin. Recrystallization from 3:5 acetone-hexane produces 1.51 g (43%) of microcrystalline p-2-hydroxyethyl-calix(4)arene phenol-oxygen-tosylate (XLIII).

(e) A solution of triphenylphosphine dibromide, prepared from 6.5 g (25 mmol) of triphenylphosphine and Br_2 (Schaefer et al., 1973), in 150 ml of dry acetonitrile is treated with a solution of the product from step (d), prepared from 3.25 g of the product of step (c), in 50 ml of acetonitrile. The mixture is stirred for 2 h at room temperature and filtered, and the solvent is removed by evaporation to leave a sticky orange oil. This is stirred with 250 ml of 95% ethanol for 8 h, and 3.10 g (78%) of p-2-bromoethyl-O-tosyl-calix(4)arene XLIV is collected as a white powder by filtration.

Q. XLVI (R_1= -OH, R_2= $-CH_2CH_2PO_3H$, R_3=R_5= H, R_4= $-CH_2-$, n=4)

Calix(4)arene derivative XLVI is prepared from p-2-bromoethyl derivative XLIV (Example P) by

modification of the method used to make XXXIX from p-chloromethyl-calix(4)arene (Example O).

Purified bromide XLIV (1 mmol) is refluxed in $P(OEt)_3$ (10 ml) overnight under nitrogen atmosphere. After removal of excess phosphite at high vacuum (100°C,0.1 mm Hg), the residue (diethyl phosphite XLV) is added to a mixture of DMSO (5 mL) and 6 N NaOH (1 ml) under nitrogen and heated at 100°C overnight, thus removing the tosylate groups. After removal of DMSO under high vacuum (100°C, < 0.1 mm Hg), the residue is diluted with hot water (25 ml) and acidified with conc. HCl to give upon cooling a precipitate which is then collected by filtration. Recrystallization of the solid from methanol provides purified XLVI.

R. XLVII. (R_1= -OH, R_2= $-CH_2SO_3H$, R_3=R_5= H, R_4= $-CH_2-$, n=4)

To a solution of p-chloromethyl-calix(4)arene (XXVII, Example O; 2.5 mmol) in 95% ethanol (10 ml) is added at room temperature an aqueous solution of Na_2SO_3 (2 M, 11 mmol). After refluxing overnight, the solvent is removed by distillation until a precipitate forms. The precipitate is collected by filtration, washed with cold, saturated aqueous NaCl, and then suspended in a minimum of water and passed through a column of Amberlite IR-120 resin in water. The UV-active fractions containing product are concentrated under vacuum and the residue is recrystallized from methanol to give purified XLVII.

S. XLIX. (R_1= -OH, R_2= $-CH_2CH_2SO_3H$, R_3=R_5= H, R_4= $-CH_2-$, n=4)

Calix(4)arene derivative XLIX is prepared from bromoethyl derivative XLIV (Example P) by applying

sequentially the sulfonation method of Example R to give XLVIII, the hydrolysis step of Example Q to remove the tosyl group, and the Amberlite IR-120 step of Example R to produce sulfonic acid XLIX.

T. LII. (R_1= -OAc, R_2= t-butyl, R_3=R_5= H, R_4= -CH(OH)-, n=4)

Calixarene LII is made via known calixarene LI (Gormer et al; 1990: R_1= -OAc, R_2= t-butyl, R_3=R_5= H, R_4= -C(=O)-, n=4) as follows.

A solution of 1.5 g (2.3 mmol) of p-tert-butyl-calix(4)arene (XXV) is refluxed in a solution of acetic anhydride (37 ml) and conc. sulfuric acid (0.1 ml). The reaction mixture is then added to 300 ml of ice water, producing an oil that slowly crystallizes. The crystalline solid is collected, washed several times with water, and dried with petroleum ether, giving the O-acetyl-p-tert-butyl-calix(4)arene (L) as white crystals.

To a three-necked round-bottom flask equipped with a condenser, stirrer, and addition funnel, are added 1.2 g (1.5 mmol) of O-acetyl-p-tert-butyl-calix(4)arene in 70 ml of acetic anhydride. To this is added dropwise a solution of 3.5 g chromium(IV)oxide in a mixture of acetic anhydride (15 ml) and acetic acid (5 ml) at 20°C with stirring, and the reaction is stirred at 140°C for 8 h. After cooling, the reaction mixture is added to 600 ml of ice-water and allowed to stand for 12 h. The resultant yellow precipitate is collected and washed with water. Recrystallization from methanol yields the purified keto derivative LI (O-acetyl-p-tert-butyl-calix(4)arene, R_4= -C(=O)-) (Gormer et al., 1990). m.p. 305°C.

To the keto derivative (1 mmol) from the previous step, dissolved in absolute ethanol (10 mL), is added $NaBH_4$ (8 mmol) in small portions at room temperature under nitrogen atmosphere. After reduction of the keto-group is complete, as judged from the disappearance of the carbonyl band at 1670 cm^{-1} observed by infrared spectroscopy, acetic acid (1 ml) is added dropwise and the resulting mixture is stirred for 1 h. The solvent is removed under high vacuum (< 0.1 mm Hg), and the resultant solid is refluxed in methanol (5 ml) for 20 min. After removal of solvent, the residue is purified by silica chromatrography, yielding purified hydroxymethylene-bridged, O-acetyl-p-tert-butyl product LII.

U. LIII. (R_1= -OH, R_2=R_3=R_5= H, R_4= -CH(Cl)-, n=4)

Derivative LII (Example T; 0.5 mmol) is refluxed in $SOCl_2$ (5 ml) under nitrogen atmosphere. After evolution of SO_2 has ceased, excess $SOCl_2$ is removed by distillation under high vacuum (< 0.1 mm Hg). To the residue is added THF (5 ml), and distillation is repeated to remove residual $SOCl_2$, yielding chloro-derivative LIII.

V. LVI. (R_1= -OH, R_2=R_3=R_5= H, R_4= -CH(CO_2H)-, n=4)

(a) A reaction mixture containing chloro-derivative LIII (1 mmol) and NaCN (1.1 mmol) in DMSO (10 ml) is heated at 80°C under nitrogen for 6 h. The mixture is then poured in ice-water (50 ml), acidified with 3 N HCl, and the resultant precipitate is collected by filtration. The filtrate is added to a mixture of DMSO (25 ml) and conc. aqueous HCl (5 ml) and refluxed overnight. After dilution with

water (100 ml) at room temperature, the resultant precipitate is collected by filtration and recrystallized from $CHCl_3$/methanol to afford LV.

(b) To remove the p-tert-butyl groups, the product from the previous step is added in small portions under nitrogen atmosphere to a hot (60°C) toluene suspension (50 ml) of $AlCl_3$ (10 mmol). After stirring overnight, the mixture is cooled to 0°C, and 1 N HCl (100 ml) is added dropwise. After the addition, the organic phase is separated and concentrated in vacuo. Recrystallization from $CHCl_3$/methanol affords purified LVI.

W. LVII. (R_1= -OAc, R_2= t-butyl, R_3=R_5= H, R_4= $-CH(CH_2CH=CH_2)-$, n=4)

Chloro-derivative LIII (Example U) is dissolved in a minimum amount of THF, and the mixture is added dropwise to a stirred, cold (-78°C) solution of THF containing $(CH_2CH=CH_2)_2CuLi$ (0.2 M, 3 mmole). The suspension is then allowed to warm to room temperature. After overnight stirring, the suspension is extracted with a 3:1 mixture (5 ml) of saturated NH_4Cl and saturated NH_3 solutions. The organic phase is dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The residue is purified by silica chromatography to provide the O-acetyl-p-tert-butyl allyl derivative LVII.

If desired, removal of the acetyl and t-butyl groups is achieved as in part b of the following Example.

X. LVIII. (R_1= -OH, R_2=R_3=R_5= H, R_4= $-CH(CH_2CO_2H)-$, n=4)

(a) Calixarene LVII (1 mmol) in CH_2Cl_2 (10 ml) is ozonized at -78°C until the reaction mixture turns

blue. Formic acid (2 ml) and then hydrogen peroxide (1 ml) are added, and the resultant mixture is allowed to warm to room temperature while being purged with nitrogen. The mixture is then refluxed overnight, after which the solvent is revoved under vacuum, yielding LVIIa.

(b) Removal of the acetyl groups is achieved by refluxing the O-acetylated product (0.2 mmol) from the previous step in a mixture of methanol (4 ml) and 6 N NaOH (1 ml) overnight. After removal of solvent under vacuum, the residue is diluted with water (10 ml), acidified to pH 2. The resultant precipitate is collected by filtration and recrystallized from $CHCl_3$/hexane provides. De-t-butylation is then effected according to step b of Example V, yielding purified LVIII.

Y. LXII. (R_1= $-O(CH_2)_3SO_3Na$, R_2= H, lower alkyl, R_3=R_5= H, R_4= $-CH_2-$, n=4)

Calix(4)arene LXII is prepared as described (Shinkai et al., 1989).

Calix(4)arene XXVI (1.54 mmol) is dissolved in THF (100 ml) at 50°C under nitrogen atmosphere. After cooling, sodium hydride (1.20 g, 30 mmol; 60% dispersion in oil) is added and the mixture is stirred until evolution of hydrogen ceases (~1 h). Propane-1,3-sulfone (2.26 g, 18.5 mmol) is then added dropwise and the mixture is stirred at room temerature for 24 h. Remaining NaH is decomposed by addition of methanol, after which the solvent is evaporated under reduced pressure, and the residue is dissolved in hot water (500 ml). Any insoluble material is removed by centrifugation. The product is then precipitated by the salting-out method with

sodium acetate to give purified LXII (10% yield). m.p. > 300°C.

Z. LXIV. (R_1= -O(CH_2)$_3$$SO_3$Na, R_2= SO_3Na, R_3=R_5= H, R_4= -CH_2-, n=4)

To a mixture of DMSO (10 ml), p-sulfonyl-calix(4)arene (XV, Figure 8; 1 mmol), and 6 N NaOH (1 ml) is added propane-1,3-sulfone (9 mmol), and the resultant reaction mixture is heated at 60°C overnight. After removal of solvents under vacuum (< 0.1 mm Hg), the solid residue is diluted with a minimum amount of water and then added dropwise to 100 ml of ethanol with stirring. The resultant precipitate is collected by filtration, and the steps of dilution in a minimum of water and dropwise addition to 100 ml of ethanol are repeated once. The precipitate is collected by filtration and recrystallized from methanol/CH_3CN provides purified LXIV.

Example 3

Preparation of Aryl-Bridged Macrocyclic Compound

A. VII. Mixed naphthyl/Phenyl Macrocycle

Chromotropic acid, disodium (10 g) in 55 ml of water was treated with 22 ml of 30 ml 37% HCl. To this solution, 1,2-benzenedimethanol (5 g) in 55 ml of acetic acid was added and this reaction was carried at reflex for 6 hours. After filtration of the resultant mixture, acetonitrile (500 ml) was added to precipitate the crude product and collected it by filtration. The crude compound was further purified by column chromatographic purification on LH-20 resin and elution with ethanol.

B. LXXV. Napthyl-Phenyl Macrocycle (n = 2 napthyl + 2 phenyl)

Mixed macrocycle LXXV is prepared using the strategy outlined by de Mendoza et al.

Chromotropic acid (III, Figure 3A; 10 mmol) and 2,5-dihydroxymethyl-3-tert-butyl-phenol (LXXII; 1 mmol) is heated at 100°C overnight in the presence of conc. HCl (5 ml). After removal of solvent under high vacuum (100°C, < 0.1 mm Hg), the residue is dissolved in a minimum amount of water and eluted through a column of Sephadex LH-20 in water. The isolated product (LXXIII; 0.6 mmol), which contains two chromotropic acid units and one phenol unit, is again heated at 100°C overnight in the presence of conc. HCl (2 ml) and 2,5-dihydroxymethyl-3-tert-butyl-phenol (LXXII; 0.6 mmol). The product (LXXIV; 0.05 mmol), isolated using a Sephadex LH-20 column, is dried under vacuum and then heated at 80°C for 6 h in conc. sulfuric acid (1 ml) under nitrogen atmosphere. After dilution with cold water (5 ml) and treatment with charcoal (100 mg), the resulting mixture is filtered, and most of the water in the filtrate is removed in vacuo (< 0.1 mm Hg). The residue is dissolved in hot, saturated aqueous NaCl. Upon cooling to 0°C, a precipitate forms. The precipitate is filtered, dissolved in a minimum of water, and eluted through a column of Amberlite IR-120 in water. The fractions containing pure product are combined and lyophilized, yielding purified LXXV (0.03 mmol).

Example 4

Cytotoxicity in Proliferating Cells

A panel of human cell lines was used to check the toxicity of the drugs. including: KB

(nasopharyngeal carcinoma), $HeLaS_3$ (cervical epithelial carcinoma), PLC (hepatocarcinoma), $HepG_2$ (human hepatocarcinoma) $HepG_2T_{14}$ (hepatocarcinoma transfected with HBV), WI38 (normal human lung fibroblast), BT549 (breast cancer), SW480 (breast cancer), and A549 (lung cancer).

5×10^4 cells were plated in each well of a 24 well multi-dish in 1 ml of RPMI-1640 containing 5% FCS and P/S. On the second day after plating, one of the fifty test compounds given in Table 3 was added to the cells, at concentrations between 1-100 μg/ml. Three days later, the medium was removed and the cells were stained with Commassie Blue in 40% methanol and 7% acetic acid. The results are discussed in Section II above.

Example 5

Inhibition of HSV Activity: Cytopathic Effect

Vero cells were maintained in RPMI-1640 medium supplemented with 5% fetal calf serum, 100 Units of penicillin per ml and 100 μg of streptomycin per ml at 37°C in a humidified incubator containing 7% CO_2. The HSV strains HSV-1 (Kos-1) and HSV-2 (333) were used.

1×10^5 Vero cells were plated in each well of a 96 well microtitre plate in 0.2 ml RPMI-1640 medium containing 5% FCS and 0.1% methyl cellulose (15 cps). After overnight incubation, and cell doubling, the medium was aspirated and replaced with 100 μl of the same medium containing 2% FCS, and 50μl control or drug solution to a final drug concentration of 10μg/ml and 50 μl virus, containing about 3 PFU/cell, i.e., 6×10^5 PFU/well, of HSV-1 or HSV-2.

The cells were cultured for 24 hours at 37°C, at which time cytopathic effects are clearly visible. In the absence of viral infection, the cells form an even monolayer of fibroblast cells. With viral infection, the cells form a suspension of round cells, followed by cell clumping, whose appearance is easily distinguishable from normal fibroblast cells. If no detectable cytopathic effect was produced, the test was repeated with 10 μg/ml. A parallel set of cells without virus inoculation were done as a control for cytotoxicity to Vero cells.

Table 1 above shows the structures of the compounds which were tested, and Table 3, column 2, the compounds which protected the cells from cytopathic effect (+).

Example 6

Inhibition of HSV Activity: Plaque Reduction

Vero cells were maintained in RPMI-1640 medium supplemented with 5% fetal calf serum, as in Example 5. 4×10^5 Vero cells were plated in a 24-well plate, in 1 ml RPMI-1640 medium containing 5% FCS and 0.1% methyl cellulose (15 cps). After overnight incubation, and cell doubling, the medium was aspirated and replaced with 100 μl of the same medium containing 2% FCS, which contained 50 μl control or drug solution to a final drug concentration of 0.25, 2.5, 5, 10, or 20 μg/ml and 50 μl virus, containing about $1x10^3$ PFU/ml, i.e., 50 PFU/well, of HSV-1 or HSV-2, as in Example 5.

After 2 hrs. at 37° absorption the virus and the drugs were removed and the cells were washed with PBS and 0.5 ml of 1% methylcellulose (4K cps) in RPMI-1640 + 2% FCS + penicillin/streptomycin (P/S) was

added. Two days later, the media were removed. The cells were stained with 0.8% crystal violet in 50% ethanol. The plaques formed were counted and the percentage of inhibition was calculated by dividing by the plaques formed in control. ED_{50} values, indicating the concentration of drug needed to produce 50% inhibition of viral plaques, were calculated assuming a linear dose response for viral plaque inhibition. The calculated IC_{50} values are given in Tables 3 and 4 above.

Example 7

Inhibition of HSV Activity: Viral Yield Inhibition

1×10^6 HeLa S_3 were plated in 25 T flasks in 5 ml RPMI-1640 + 5% FCS + P/S. 24 hours later, the medium was aspirated and replaced with 6×10^6 PFU HSV-1 or HSV-2, and serial dilutions of selected KY compounds, at 10, 5, 2.5, 1.25, and 0.625 µg/ml drug. After growth at 37°C for 24 hours in 2 ml of RPMI-1640 containing 2% FCS and P/S, the cells were frozen at -70°C until the time for titration. The cells were freeze/thawed 3 times to release virus from the cells, and serially diluted 10 fold.

$1X\ 10^5$ Vero cells were plated in each well of 24 well multi-dish in 1 ml RPMI-1640 + 5% FCS + P/S + 0.1% methylcellulose (15 cps). On the second day, after removal of the medium, the 10 fold serially diluted virus in 100 µl was added in duplicate. After 2 hours incubation at 37°C, the virus was removed and 0.5 ml methycellulose (4K cps) in RPMI-1640 and 2% FCS + P/S was added. Two days later, the medium was removed. The cells were stained in 0.8% crystal violet in 50% ethanol. The plaques formed

were counted and the titer was calculated from the fold of dilutions.

The reduction in virus yield, as a function of KY compound concentration, is seen in Figures 29A and 29B for KY-1 and KY-2.

Example 8

Activity Against Drug-Resistant Strains of HSV-1 and HSV-2

The following strains of HSV-1 and HSV-2 virus were used: KOS, a wild type HSV-1 virus; KOS (PMEA) and KOS (PFA), both drug-resistant HSV-1 viruses having a DNA polymerase mutation; 333, a wild type HSV-2 HSV-2 virus, and 333 (DHPG), a drug-resistant HSV-2 virus having a thymidine kinase mutation.

Inhibition of viral yield was by KY-1, acyclovir (ACV), DHPG, PFA, and PMEA was examined in each of the five HSV strains substantially as described in Example 7. Briefly, Hela S_3 were plated in 25 T flasks in culture, and 24 hours later, the medium was aspirated and replaced with 6×10^6 PFU of the selected HSV strain, and serial dilutions of KY-1, ACV, DHGP, PFA, and PMEA. After growth at 37°C for 24 hours in 2 ml of RPMI-1640 containing 2% FCS and penicillin and streptomycin (P/S), the cells were frozen at -70°C until the time for titration. The cells were freeze/thawed 3 times to release virus from the cells, serially diluted 10 fold, and the serial dilutions were added to Vero cells in culture. After 2 hours incubation at 37°C the virus was removed and 0.5 ml methycellulose (4K cps) in RPMI-1640 and 2% FCS + P/S was added. Two days later, the medium was removed. The cells were stained in 0.8% crystal violet in 50% ethanol. The plaques formed were counted and the titer was calculated from the

fold of dilutions. From the drug dose response, the concentration of each drug required to effect a 90% inhibition of virus yield, the IC_{90} concentration was determined. These values are shown in Table 5 above.

Example 9

Inhibition of RSV Activity

Assays to assess the antiviral activity of KY- and Y- compounds in tissue culture were performed in 96-well flat-bottom tissue culture plates (Falcon 307), using conditions similar to those used in the cytotoxicity assays described above. In these assays, compound was tested in quadruplicate by serially diluting the compound in 2% FCS-MEM using serial two-fold dilutions (0.05 ml/well). A 0.05 ml volume of the appropriate virus containing approximately 100 median tissue culture infectious doses ($TCID_{50}$) was then added to all wells except those set aside as antiviral and tissue control wells. Next, approximately 3×10^3 HEp2 cells (0.1 ml) were added to each well. Control wells containing antiviral and no virus (antiviral control), containing virus but no antiviral (virus control), or containing medium without virus or antiviral (tissue control), were included in each test. The challenge virus was then back titrated. All assay plates were incubated at 35°C for 5 to 7 days in a 5% CO_2 incubator. When virus control wells exhibited 70% to 100% CPE including syncytia, all wells were observed. The median efficacious concentration (IC_{50}) was calculated after determining the final concentration of antiviral in the last wells in each set of quadruplicate rows exhibiting <50% CPE compared to the CPE in virus control wells.

The ED_{50} values calculated for each of the compounds tested are shown in Table 5.

Example 10

Activity against HSV virus: Topical activity against in vivo ocular cultures of HSV-1

New Zealand white rabbits were acclimated for a minimum of two days prior to inoculation to allow the animals to accommodate to conditions in the vivarium facility. After the accommodation period, animals received a slit lamp ocular examination to exclude any animals with preexisting anterior segment ocular defects. Animals were bilaterally inoculated topically with an 80 µl drop of Minimal Essential Medium (MEM; Gibco) containing 10^5 pfu/ml McKrae strain HSV-1; eyes were massaged for 30 seconds. Animals were replaced individually in cages.

On day 4 post inoculation (PI), animals were evaluated by slit lamp microscopy. Corneal epithelial, iris, and conjunctival disease were graded on an increasing scale of severity from 0+ to 4+. After evaluation, animals were divided into 4 groups of 5 animals with matched corneal, stromal and conjunctival involvement. Topical therapy was initiated immediately after animal grouping. Therapy groups included:

Group #1: 5 rabbits, Y-1 topical therapy (6.25 µg/50 µl) 5x/day for 4 days;

Group #2: 5 rabbits, Y-1 topical therapy (12.5 µg/50 µl) 5x/day for 4 days;

Group #3: 5 rabbits, Y-1 topical therapy (18.75 μg/50 μl) 5×/day for 4 days;

Group #4: 5 rabbits, placebo therapy (sterile water) 5×/day for 5 days.

The concentration of Y-1 for the ascending dose tolerance study were based upon the ED90 concentrations determined in the virus yield or CPE assays. Group 1 received topical eyedrop therapy containing 6.25 μg/50 μl [one-half of the ED90 concentration]; Group 2 received eyedrop therapy containing 12.5 μg/50 μl [the ED90 concentration]; Group 3 received eyedrop therapy containing 18.75 μg/50 μl [1.5 times the ED90 concentration]. All Y-1 doses were formulated to contain these concentrations in a volume of 50 μl (a standard eye drop).

Topical therapy with 0-19 μg Y-1 in 50 μl was initiated on day 4 post-inoculation (PI) and continued to day 7 PI. All animals received daily ocular slit lamp evaluations from day 3 through day 7 PI. The ocular HSV-1 induced disease severity was recorded daily.

Eyes of all animals were additionally sampled for the presence of infectious HSV-1 on days 0 (pre-inoculation), 3, 5, and 7 PI. Briefly, tear film was obtained by swabbing the lower and upper conjunctival sacs and retaining the swab in the nasal fornix for 10 seconds. The swabs were eluted individually in Hank's Buffered Saline (HBSS, Gibco Laboratories). Fifty microliter aliquots of the virus-HBSS eluate was adsorbed onto confluent HFF cell monolayers for 5 minutes. Monolayers were hydrated with Minimal

Essential Medium (MEM; Gibco Laboratories), incubated at 37°C and observed daily for two weeks to detect cytopathology consistent with HSV infection (HSV CPE). Cultures not exhibiting HSV CPE were blind passaged to confirm negativity.

On day 7 PI (sacrifice), the corneal epithelium was scraped from the eyes and HSV was recovered on HFF cell monolayers. Corneal epithelial co-cultures were evaluated daily by inverted light microscopy. Cultures not exhibiting HSV CPE were blind passaged to confirm negativity.

Clinical efficacy of the three Y-1 concentrations used in single-agent therapies were compared to placebo therapy. Virus recovery during and after topical therapy with the Y-1 formulations were compared to each other and to placebo therapy, as illustrated in Figures 33(A-D).

Example 11

Inhibition of Influenza A Activity

The anti-influenza A activity of KY compounds was evaluated as described in Example 9, except that MDCK cells (kidney cell line) was used for infection in vitro by influenza virus (strain A/Taiwan).

Example 12

Inhibition of HIV-Induced Cell Fusion

Human CD_4^+ indicator cells (VB) and chronically infected H_9 cells were maintained in RPMI-1640 medium supplemented with 5% fetal calf serum, 100 Units of penicillin per ml and 100 μg of streptomycin per ml at 37°C in a humidified incubator containing 7% CO_2. The HIV strains that were used were HTLV-III_B and RF-

II strains obtained from the National Institutes of Health (Bethesda, MD).

For the fusion assay, serial dilutions between 1:2 and $1:2^8$ of a selected KY compound, 1 mg/ml in PBS were made in a 96 well round bottom plate. The diluted KY compound was transferred to a 96 well flat-bottom plate. To each well was added 25 µg chronically infected H_9 cells (at 2×10^6, cells/ml), or cells chronically infected with RF-II strain HIV, followed by incubation at 37°C for 45 minutes. To each well was then added 25 µl VB cells (about 5×10^4 cells), and the cells and virus isolates were cocultured for 18 hours in a humid 5% CO_2 atmosphere. The extent of syncytia formation was scored under phase microscopy, and the concentration which completely inhibited syncytia formation (ED_{100}) was recorded. The results are given in Table 7.

Example 13

Effect of Topical Administration on Genital HSV Infection

A. Virus and Viral Inoculation

The MS strain of HSV-2 was utilized for the experimental animal infection. Female Hartley strain guinea pigs (Charles River Breeding Laboratories, Kingston, NY) weighing 250-300 g were inoculated intravaginally with 2.0×10^5 plaque-forming units of HSV-2 one hour after being swabbed for removal of vaginal secretions.

B. Treatment of Guinea Pigs

In the first study, groups of 10 guinea pigs were treated topically (0.1 ml intravaginally + 0.1 ml on external genital skin) three times daily (approximately every eight hours) for seven days

beginning 6h or 48h after inoculation with HSV-2. Groups of three uninfected animals were treated in a similar manner to assess any skin irritation.

In a second study, groups of 8-10 animals were treated three times daily with topical formulations of 2% or 6% KY-1 or Y-1, with treatment beginning either 6 or 24 hours following viral inoculation, as indicated in Table 8B. Formulations of KY-1 or Y-1 were prepared by dissolving the compound in a 1.5% methyl cellulose solution such that final concentration of compound was 2% or 5% (wt/wt). Control animals were given either no treatment (N=10 animals) or treatment with placebo (1.5% methylcellulose solution).

C. Sample Collection and Virus Assays

To determine the effect of treatment on vaginal viral replication, swabs of vaginal secretions were obtained on days 1, 3, 5, 7 and 10 after HSV inoculation, placed in a tube containing 2.0 ml of media, vortexed and frozen at -70°C until titrated for HSV-2. When all samples were collected, they were thawed, diluted serially and HSV-2 titers determined using rabbit kidney cells in a microtiter CPE assay.

D. Evaluation of Efficacy

To determine the effect of therapy on the development and spread of external genital lesions, lesion severity was scored on a 0-5+ scale through the primary infection period (19-21 days). Lesion score-day areas and virus titer-day areas under the curve, and peak lesion scores and peak virus titers between untreated and placebo-treated or placebo-treated and drug-treated animals were compared using

the Mann-Whitney U range sum test. A p-value of 0.05 or less was considered significant. The results are discussed with reference to Tables 10 and 11 in Section IV above.

Animals were scored daily for 19 days following inoculation for presence of lesions and severity of lesions (on a 0-5+ point scale). Lesion scores were tabulated as area under the curve of daily lesion score vs. time (days) and peak lesion score observed. Data are presented in Table 11. A known antiviral agent, acyclovir (ACV) was administered in a 5% formulation to 8 animals as a positive control in the study.

Example 14

Inhibition of HSV-1 Binding to Vero Cells

Vero cells were maintained in RPMI-1640 medium, as described in Example 5. After overnight incubation, and cell doubling, the medium was aspirated and replaced with 100 µl of medium containing 2% FCS composed of 50µl control or drug solution to a final drug concentration of 10µg/ml and 50 µl virus, containing about 3 PFU/cell, i.e., 6 x 10^5 PFU/well, of H^3-labeled HSV-1. At time intervals of 5, 30, 60, 120, and 240 minutes, cells were removed from the suspension, washed two times with PBS, and assayed for bound virus (cpm ^3H). The results are given in Figure 30, where the control virus binding is indicated by solid circles, and the drug-inhibited binding, by open rectangles.

Example 15

Effect of Drug/Virus Exposure on HSV Inhibition

Vero cells were maintained in RPMI-1640 medium, as above. After overnight incubation, and cell doubling, the medium was aspirated and replaced with 100 µl of medium containing 2% FCS. In one group of wells, serial dilutions of KY-1 compound, between 0.625 and 10 µg/ml drug were added in 50 µl, together with 50µl of HSV-1 virus suspension, 5×10^6 PFU per well. The cells were incubated for 2 hours at 37°C, then washed with PBS and assayed for number of virus plaques, as in Example 6.

In a second group of cells, serial dilutions of the drug were added to the cells, prior to the addition of the HSV-1 virus, and the cells were incubated for 2 hours at 37°C in the presence of the virus. After washing the cells to remove free drug, virus suspension was added, 5×10^6 PFU per well. The cells were incubated for 2 hours at 37°C, then washed with PBS and assayed for number of virus plaques, as in Example 6.

In a third group of cells, 100 µl virus suspension was added to the cells, 5×10^6 PFU per well, and the cells were incubated for 2 hours at 37°C, then washed with PBS to remove unbound virus. Serial dilutions of KY-1 compound, between 0.625 and 10 µg/ml drug were added to the cells in 100 µl. The cells were incubated for 2 hours at 37°C in the presence of the drug, then washed with PBS and assayed for number of virus plaques, as above.

The numbers of plaques observed in each of the above treatment methods, expressed as percent of untreated control, are plotted in Figure 31. The solid circles indicate co-exposure of the cells to drug and virus; the solid squares, preincubation of the cells with drug before addition of virus; and the

open squares, preincubation of the cells with virus before addition of drug.

Example 16

Inactivation of HSV-1 by KY compounds

Purified HSV-1 was suspended in RPMI-1640 medium (Gibco Laboratories) containing 2% FCS, penicillin and streptomycin. To aliquots of the suspensions were added control, KY-1, or KY-217 solution, to a final drug concentration of 10 μg/ml, and a final virus particle concentration of 6×10^6 or 6×10^5 PFU/ml. The suspensions were incubated for 1 hour at 37°C, then diluted serially at 10 fold dilutions to final drug concentrations of 10, 10^0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} μg/ml drug concentrations. The serially diluted particles were then added to Vero cells for two hours, as in Example 6, and the cells examined for plaques 48 hours later. The number of plaques counted on each of two plates, for each virus and drug concentration, are given in Table 9.

Example 17

Binding of KY Compounds to HSV Proteins

A. Binding of KY compound to HSV Proteins

HSV-1 and HSV-2 viral suspensions from above, each at a concentration of about 5×10^7 CFU/ml, were incubated for 2 hours at 37°C with 5×10^5 cpm ^{14}C-labeled KY-1 (50μg/ml). Each viral suspension was divided into two aliquots and solubilized with 0.5% sodium dodecyl sulfate (SDS), with or without 1% mercaptoethanol. The four solubilized samples were fractionated on 8.5% polyacrylamide gel, and the gels developed by autoradiography, according to standard procedures. The autoradiographs of the four samples

are seen in Figure 32A, where the lanes are HSV-1, with (lane A) and without (lane B) mercaptoethanol, and HSV-2, with (lane D) and without (lane E) mercaptoethanol, with the marker proteins in lane C.

B. Identification of Binding Proteins

HSV-1 and HSV-2 virus suspensions were solubilized with SDS and fractionated on SDS-PAGE as above. Each sample was run in triplicate, corresponding to groups D, B, and C in Figure 32B. The two gels in each group were analyzed by Western blotting as follows: The gels in groups D, B, and C were first reacted with mouse monoclonal antibody specific against HSV glycoprotein gD, gB, and dC, respectively. The antibodies were obtained from Dr. S. Chatterjee from the University of Alabama. The gels were then incubated with alkaline phosphatase-labeled goat anti-mouse antibody, to label the glycoprotein in each group. The glycoprotein with bound antibody was identified by reaction with H_2O_2 in the presence of nitroblue tetrazolium and bromochloroindolephosphate, according to standard methods. The results are shown in Figure 32B.

Example 18

Inhibition of HSV By Combined Drug Exposure

A. Inhibition of HSV-1

HSV-1 particles were obtained from infected HeLa cells, as described in Example 7. $1X\ 10^5$ Vero cells were plated in each well of 24 well multi-dish in 1 ml RPMI-1640 + 5% FCS + P/S + 0.1% methylcellulose (15 cps). On the second day, after removal of the medium, the 10 fold serially diluted virus in 100 µl was added in duplicate, plus (i) a selected

concentration of GL-288 alone (up to 50 μg/ml), (ii) a selected concentration of acyclovir alone (up to 50 μg/ml); (iii) a selected concentration of acyclovir (up to 50 μg/ml) plus 25 μg/ml GL-288; or (iv) a selected concentration of acyclovir (up to 50 μg/ml) plus 50 μg/ml GL-288, to Vero cells in culture.

After 2 hours incubation at 37°C, the virus was removed and 0.5 ml methycellulose (4K cps) in RPMI-1640 and 2% FCS + P/S was added. Two days later, the medium was removed. The cells were stained in 0.8% crystal violet in 50% ethanol. The plaques formed were counted and the titer was calculated from the fold of dilutions.

The reduction in virus yield, as a function of compound concentration, is seen in Figure 34A.

B. Inhibition of HSV-2

HSV-2 particles were obtained from infected HeLa cells, as described in Example 7. Vero cells were infected with serial dilutions of the virus particles plus GL-228 alone, acyclovir alone, or acyclovir plus GL-228 as described in Section A. After 2 hours incubation at 37°C, the virus was removed, and 0.5 ml methycellulose (4K cps) in RPMI-1640 and 2% FCS + P/S was added. Two days later, the medium was removed. The cells were stained in 0.8% crystal violet in 50% ethanol. The plaques formed were counted and the titer was calculated from the fold of dilutions.

The reduction in virus yield, as a function of compound concentration, is seen in Figure 34B.

Example 19

Inhibition of HSV Activity: Cytopathic Effect

Vero cells were maintained in RPMI-1640 medium supplemented with 5% fetal calf serum, 100 Units of

penicillin per ml and 100 µg of streptomycin per ml at 37°C in a humidified incubator containing 7% CO_2. The HSV strains HSV-1 (Kos-1) and HSV-2 (333) were used.

A topical formulation of compound Y-1 was created by dissolving Y-1 in K-Y Jelly (Johnson & Johnson) at 5, 10, 15 and 20% (weight/weight) concentrations. Each of the Y-1 formulations was then dissolved and diluted in RPMI-1640 containing 2% FCS.

1×10^5 Vero cells were plated in each well of a 96 well microtitre plate in 0.2 ml RPMI-1640 medium containing 5% FCS and 0.1% methyl cellulose (15 cps). After overnight incubation and cell doubling, the medium was aspirated and replaced with 100 µl of the same medium containing 2% FCS, and 50 µl control or drug solution yielding final drug concentrations of 5, 10, 20 and 40 µg/ml and 50 µl virus, containing about 3 PFU/cell, i.e., 6×10^5 PFU/well, of HSV-1 or HSV-2.

The cells were cultured for 24 hours at 37°C, at which time cytopathic effects are clearly visible. In the absence of viral infection, the cells form an even monolayer of fibroblast cells. With viral infection, the cells form a suspension of round cells, followed by cell clumping, whose appearance is easily distinguishable from normal fibroblast cells. A parallel set of cells without virus inoculation were done as a control for cytotoxicity to Vero cells. No cytotoxicity by K-Y Jelly alone was observed. The results are shown in Table 12, where a "+" indicates full inhibition of cytopathic effects, and a "-" indicates no inhbition of cytopathic effects.

Example 20

Inhibition of HIV-Induced Cell Fusion with Y-1 Formulated in K-Y Jelly

Human CD_4^+ indicator cells (VB) and chronically infected H_9 cells were maintained in RPMI-1640 medium supplemented with 5% fetal calf serum, 100 units of penicillin per ml and 100 μg of streptomycin per ml at 37°C in a humidified incubator containing 7% CO_2. The HIV strains that were used were $HTLV\text{-}III_B$ and RF-II strains obtained from the National Institutes of Health (Bethesda, MD).

Topical formulations of compound Y-1 were prepared by dissolving Y-1 in K-Y Jelly (Johnson & Johnson) at 5, 10, 15 and 20% (weight/weight) concentrations. Each Y-1 formulation was then dissolved in PBS to a final Y-1 concentration of 1 mg/ml.

For the fusion assay, serial dilutions of each Y-1 formulation were transferred to a 96 well flat-bottom plate. To each well was added 25 μl chronically infected H_9 cells (at 2×10^6, cells/ml), or cells chronically infected with RF-II strain HIV, followed by incubation at 37°C for 45 minutes. To each well was then added 25 μl VB cells (about 5×10^4 cells), and the cells and virus isolates were co-cultured for 18 hours in a humid 5% CO_2 atmosphere. The extent of syncytia formation was scored under phase microscopy. Complete inhibition of syncytia formation was recorded as "4+". The absence of syncytia formation was recorded as "-". The results are given in Table 13.

Although the invention has been described with reference to preferred compounds and method of virus inhibition employing the compounds, it will be

appreciated that various modification and changes may be made without departing from the invention.

IT IS CLAIMED:

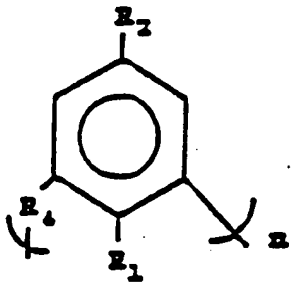
1. A method of inhibiting cell infection by an enveloped virus comprising

administering to the site of infection a therapeutically effective dose of a calix(n)arene compound which is derivatized, at its ring positions meta to the bridge attachments to the ring, with polar substituents having a terminal carboxylate, phosphonate, sulfinate or sulfonate group.

2. The method of claim 1, wherein the number of subunits in the compound (n) is 4-10.

3. The method of claim 1, wherein the calix(n)arene compound is partially oxidized.

4. The method of claim 1, wherein the calix(n)arene compound has the general structure:



wherein n = 4, 6, or 8;

R_1 is OH or =O or a mixture thereof;

R_2 is a polar substitutent with a terminal carboxylate, phosphonate, sulfinate or sulfonate group; and

R_4 is $>CH_2$ or $\geq CH$, or a mixture thereof.

5. The method of claim 4, wherein R_2 is $(CH_2)_mR_2'$, where m= 1-3, and R_2' is a sulfonate group.

6. The method of claim 5, wherein the sulfonate group is SO_3R or SO_2NRR', and R and R' are lower alkyl groups.

7. The method of claim 4, wherein R_2 is $(CH_2)_mR_2'$, where m= 1-3, and R_2' is a sulfinate group.

8. The method of claim 7, wherein the sulfinate group is SO_2R or $S(=O)NRR'$, and R and R' are lower alkyl groups.

9. The method of claim 4, wherein R_2 is $(CH_2)_m-R_2'$, where m= 0-3, and R_2' is a carboxylate group.

10. The method of claim 9, wherein the carboxylate group is CO_2R or $C(O)NRR'$, where R and R' are lower alkyl groups.

11. The method of claim 4, wherein R_2 is $(CH_2)_m-R_2'$, where m= 0-3, and R_2' is a phosphonate group.

12. The method of claim 11, wherein the phosphonate group is $PO(OR)_2$, $PO(OH)(OR)$, $PO(OR)(NRR')$, $PO(NRR')_2$, where R and R' are each H or a lower alkyl group.

13. The method of claim 1, for use in treatment of HIV, RSV, HSV-1 or HSV-2, wherein the compound is administered orally.

14. The method of claim 1, for use in treatment of HIV, RSV, HSV-1 or HSV-2, wherein the compound is administered intravenously.

15. The method of claim 1, for use in oral or parenteral administration, which further includes administering to the subject, protamine sulfate in an amount effective to inhibit anti-coagulant effects of the calix(n)arene compound.

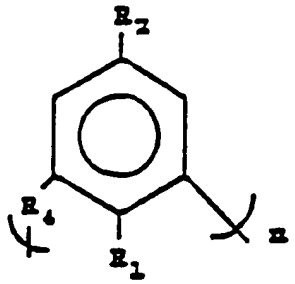
16. The method of claim 1, which further includes administering to the subject, an antiviral nucleoside analog compound.

17. A method of inhibiting infection by a sexually transmitted enveloped virus, comprising

topically administering to an area of likely sexual contact, a composition containing a prophylactically effective amount of a macrocyclic compound composed of aryl ring subunits which are connected by ring-attached bridge linkages which form a continuous chain of connected atoms making up the backbone of the macrocycle, and which contain negatively charged substituents on non-backbone atoms of the aryl subunits in a lubricating jelly.

18. The method of claim 17, wherein the composition is used in conjunction with a physical-barrier type device.

19. The method of claim 17, wherein the compound has the form:



where n = 4, 6, or 8;

R_1 is OH or =O, an alkyl or aryl ether, an ester, an acid, or a mixture thereof;

R_2 is a polar substitutent having a terminal, negatively charged substituent selected from the group consisting of a sulfonate group, a sulfinate group, a carboxylate group, and a phosphonate group; and

R_4 is $>CH_2$ or $\geq CH$, or a mixture thereof.

20. The method of claim 19, wherein at least one of the R_2 groups is =O.

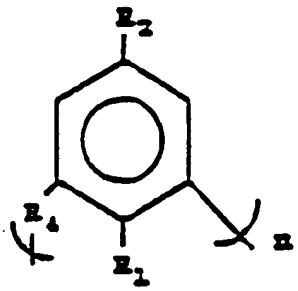
21. The method of claim 17, wherein the enveloped virus is selected from the group consisting of hepatitis delta virus, hepatitis B virus, hepatitis C virus, papilloma virus, HSV-1, HSV-2, HIV-1, HIV-2, HTLV-I, and HTLV-II.

22. The method of claim 17, where said virus is selected from the group consisting of hepatitis delta virus, hepatitis B virus, and hepatitis C virus.

23. The method of claim 17, where said virus is selected from the group consisting of HSV-1 and HSV-2.

24. The method of claim 17, where said virus is selected from the group consisting of HIV-1, HIV-2, HTLV-I and HTLV-II.

25. A calix(n)arene compound having the form



wherein n = 4-10;

R_1 is OH or =O, where at least one of the R_1 groups in the calix(n)arene compound is =O;

R_2 is a polar substituent with a terminal carboxylate, phosphonate, sulfinate or sulfonate group; and

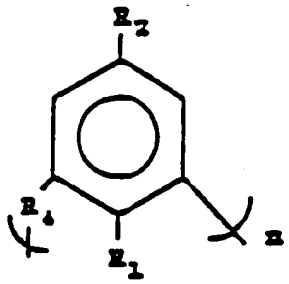
R_4 is $>CH_2$ or $\geq CH$, or a mixture thereof.

26. The compound of claim 25, wherein R_2 is $(CH_2)_m R_2'$, where m= 1-3, and R_2' is SO_3R or $SO_2NR'R''$, where R, R', and R'' are each H or a lower alkyl group.

27. The compound of claim 25, wherein R_2 is $(CH_2)_m$-R_2', where m= 0-3, and R_2' is CO_2R' or C(O)NR'R'', where R, R', and R'' are each H or a lower alkyl group.

28. The compound of claim 25, wherein R_2 is $(CH_2)_m$-R_2', where m= 0-3, and R_2' is $PO(OR)_2$ or PO(OH)(OR), where R is H or a lower alkyl group.

29. A calix(n)arene compound having the form



wherein n = 4-10; R_1 is OH; R_2 is a polar substituent with a terminal carboxylate or sulfinate group, and R_4 is $>CH_2$ or $\geq CH$, or a mixture thereof.

30. A pharmaceutical composition for inhibiting cell infection by an enveloped virus comprising

administering to the site of infection a therapeutically effective dose of a calix(n)arene compound which is derivatized, at its ring positions meta to the bridge attachments to the ring, with polar substituents having a terminal carboxylate, phosphonate, sulfinate or sulfonate group.

31. A pharmaceutical composition, for inhibiting infection by a sexually transmitted enveloped virus, comprising

a calix(n)arene compound which is derivatized, at its ring positions meta to the bridge attachments to the ring, with polar substituents having a terminal carboxylate, phosphonate, sulfinate or sulfonate group.

32. The composition of claim 31, wherein the enveloped virus is selected from the group consisting of hepatitis delta virus, hepatitis B virus,

hepatitis C virus, papilloma virus, HSV-1, HSV-2, HIV-1, HIV-2, HTLV-I, and HTLV-II.

33. A physical barrier type device for use in inhibiting infection by a sexually transmitted enveloped virus, comprising

a physical barrier-type device in combination with a composition composed of a vehicle and, dissolved in the vehicle, a macrocyclic compound composed of aryl ring subunits which are connected by ring-attached bridge linkages which form a continuous chain of connected atoms making up the backbone of the macrocycle, and which contain negatively charged substituents on non-backbone atoms of the aryl subunits.

34. The device of claim 33, wherein the device is a condom, diaphragm, or cervical sponge.

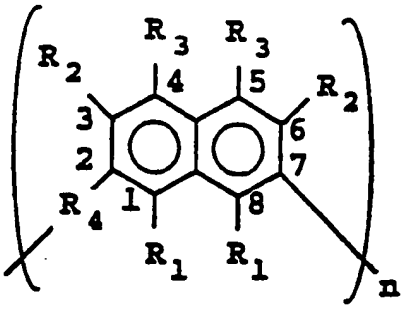
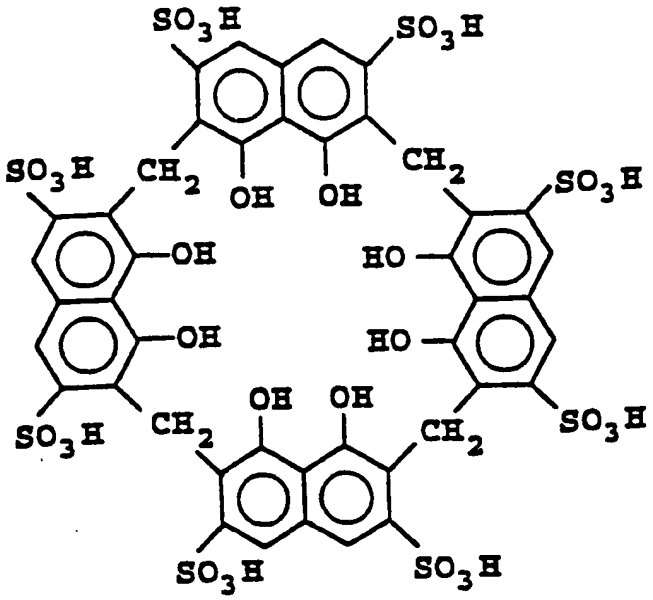
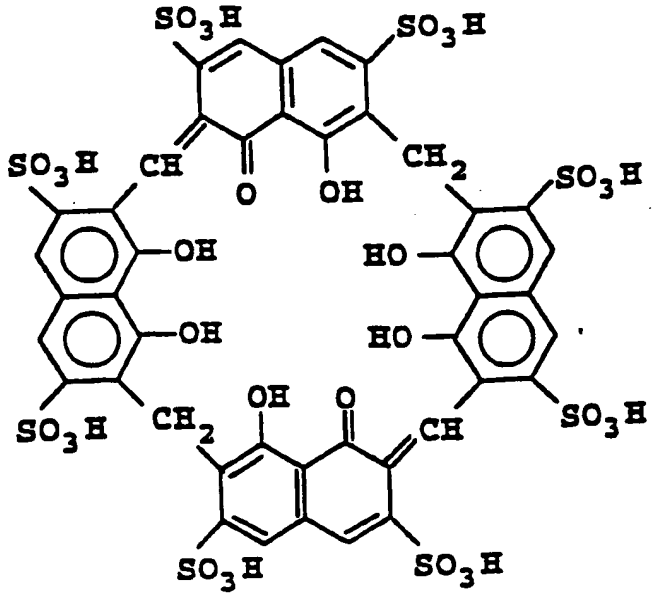


Fig. 1



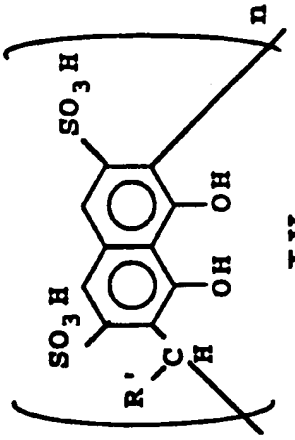
I

Fig. 2A



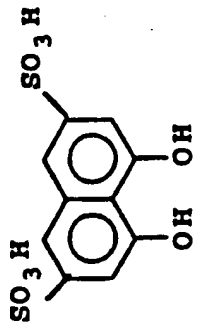
II

Fig. 2B



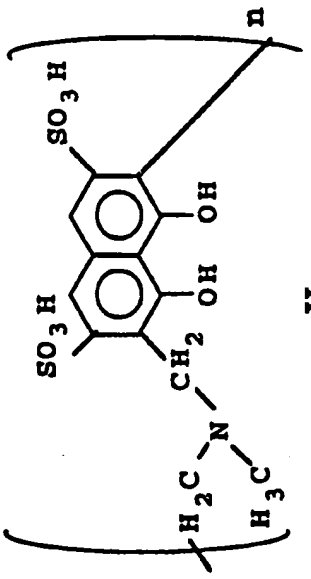
IV

+ R'-CHO

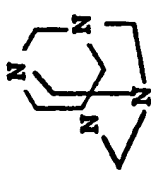


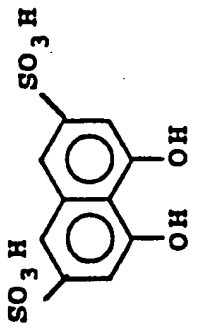
III

Fig. 3A



V

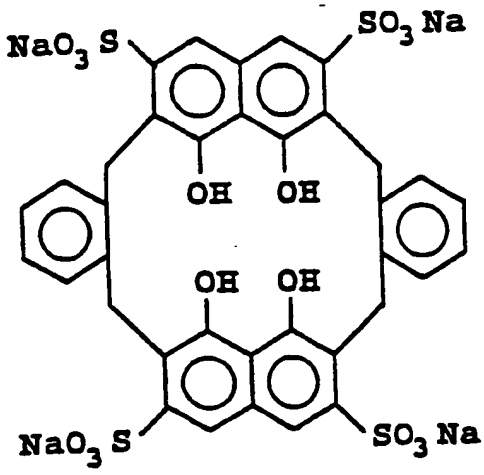




+

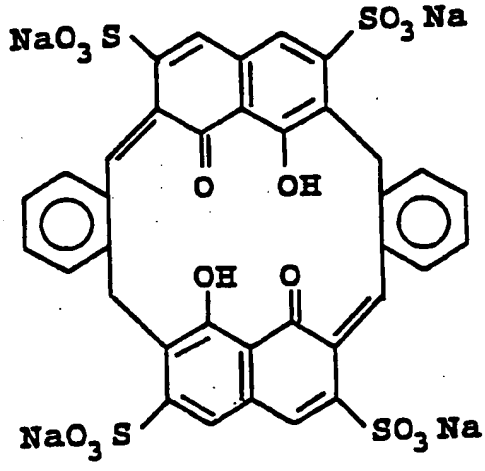
III

Fig. 3B



VI

Fig. 4A



VII

Fig. 4B

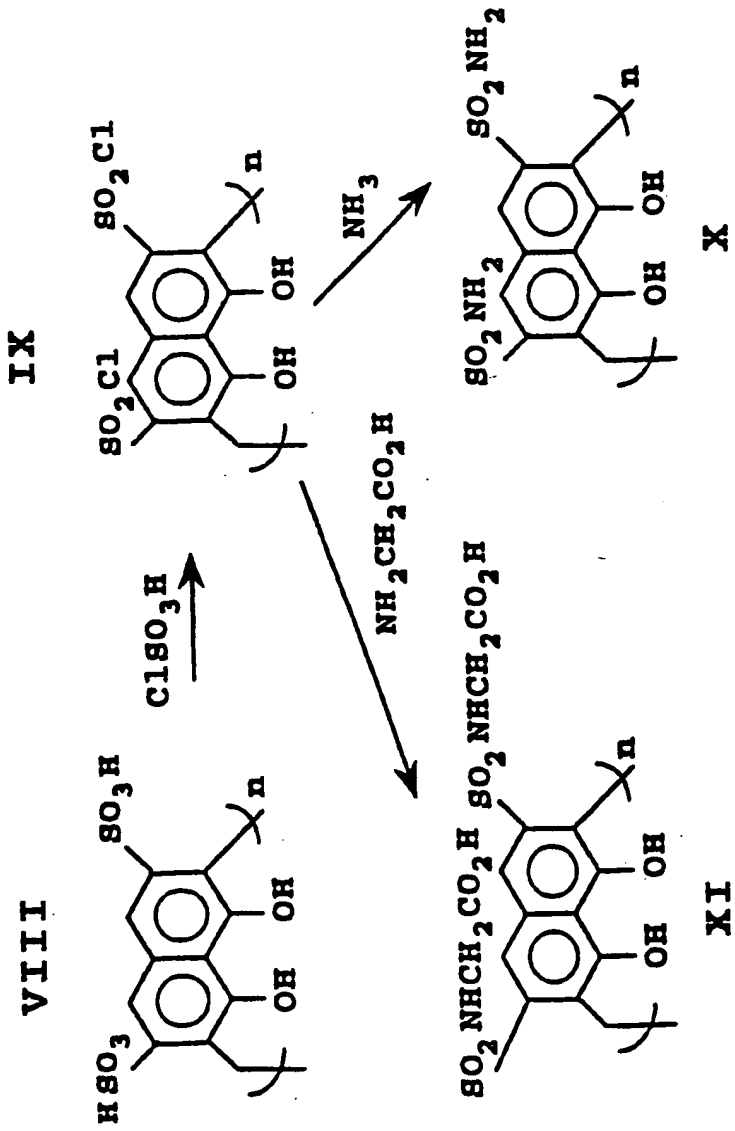

VIII
IX
X
XI
$ClSO_3H$
NH_3
$NH_2CH_2CO_2H$
HSO_3
SO_3H
SO_2Cl
SO_2NH_2
$SO_2NHCH_2CO_2H$
OH
n

Fig. 5

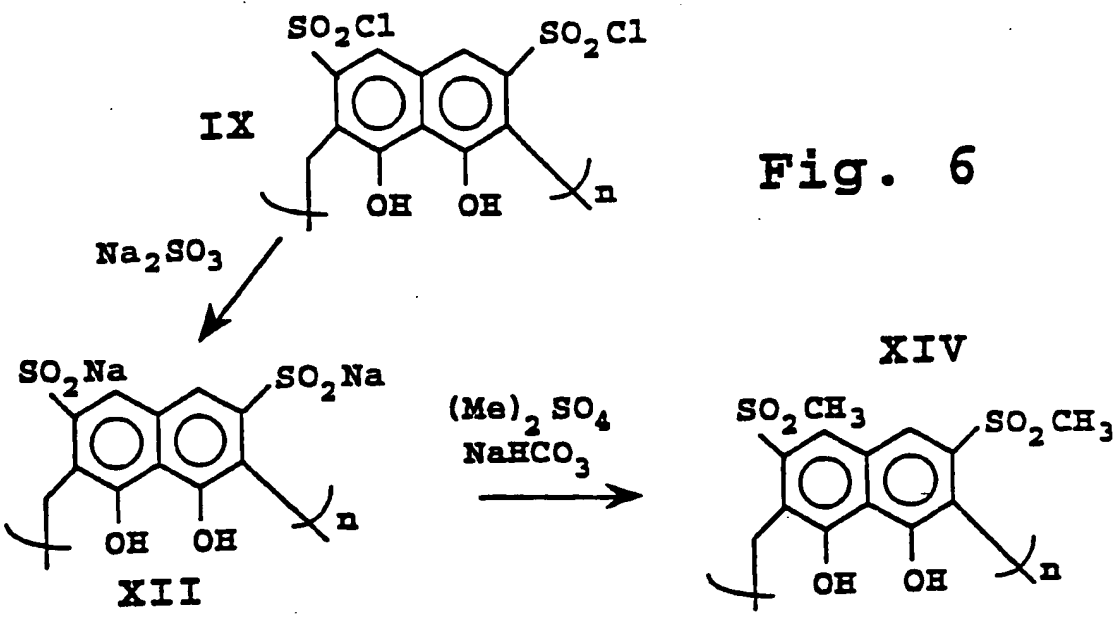


Fig. 6

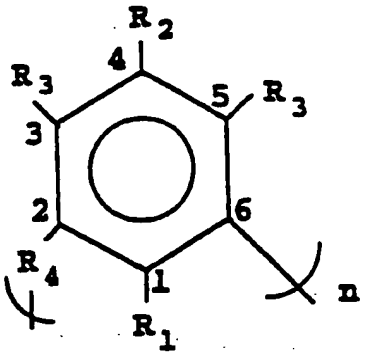


Fig. 7

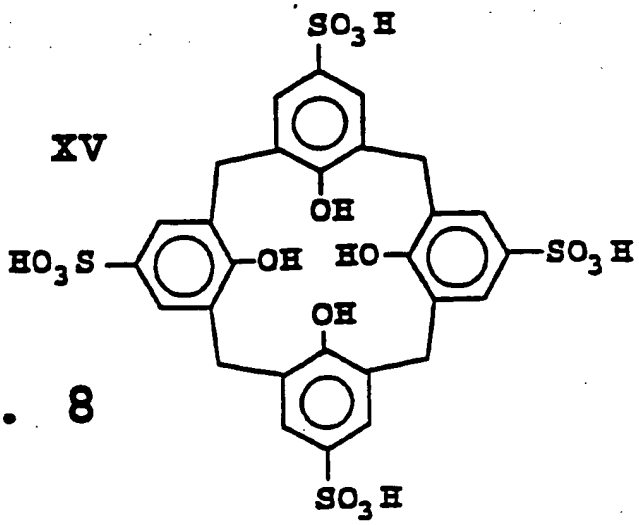
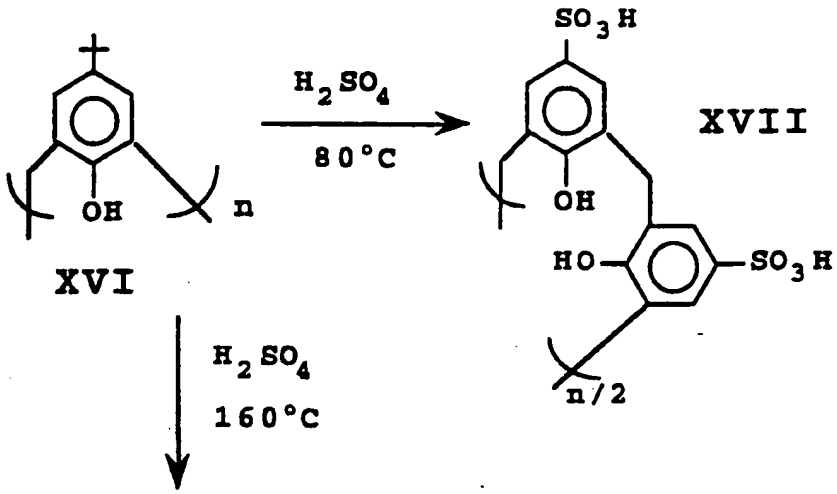
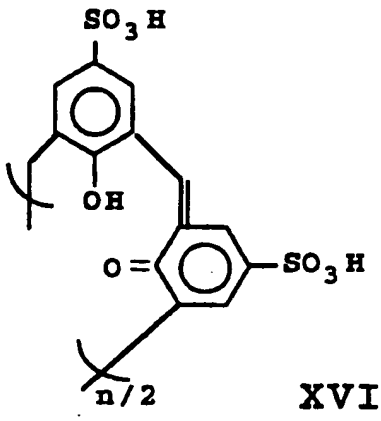


Fig. 8

Fig. 9A





XVIII

Fig. 9B

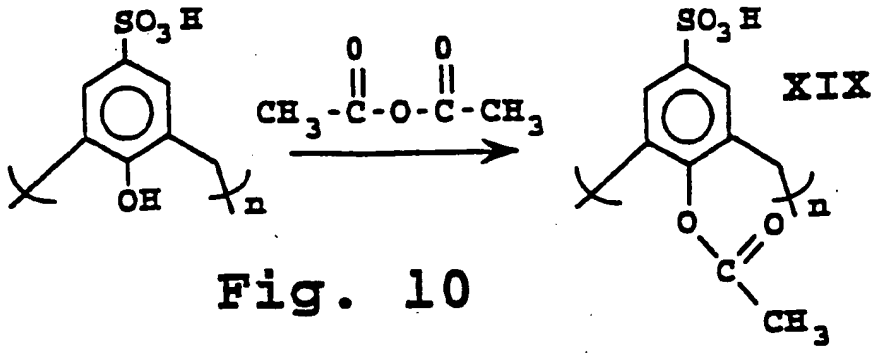

SO3H
OH
n
CH3-C-O-C-CH3
O
O
SO3H
XIX
O
O
C
CH3

Fig. 10

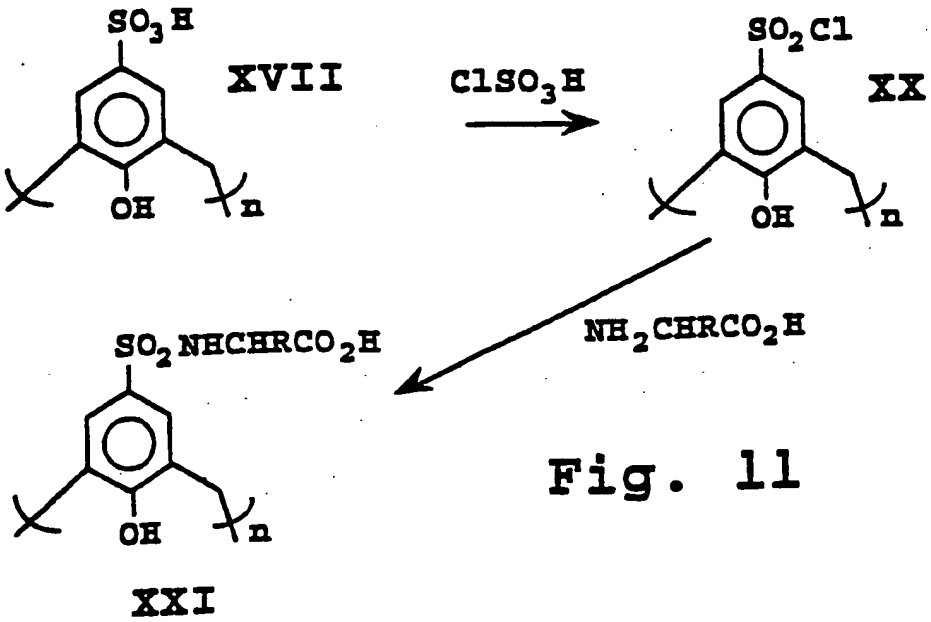

SO3H
XVII
OH
n
ClSO3H
SO2Cl
XX
OH
n
NH2CHRCO2H
SO2NHCHRCO2H
OH
n
XXI

Fig. 11

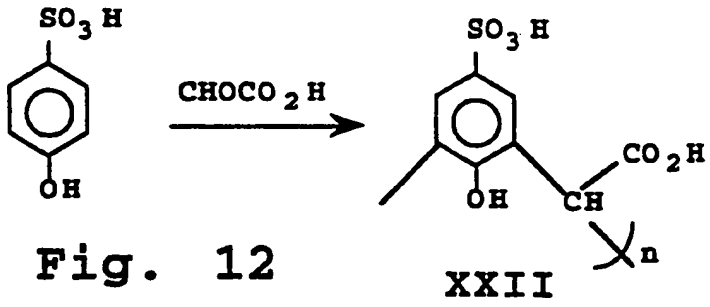
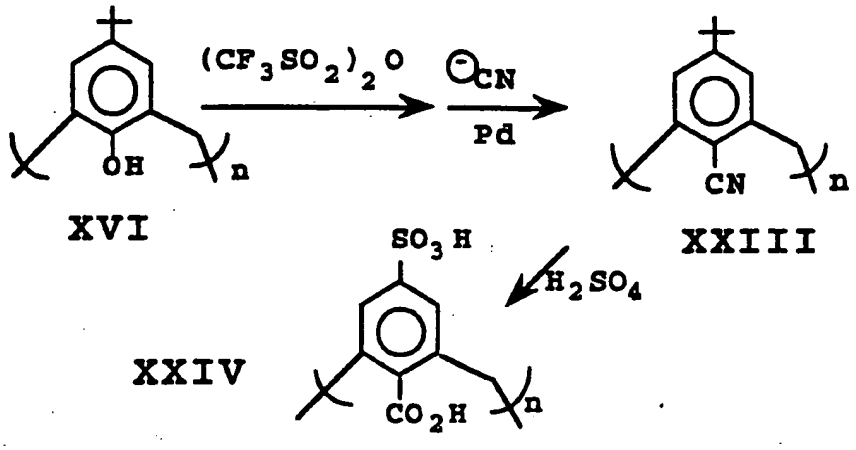


Fig. 12

XXII

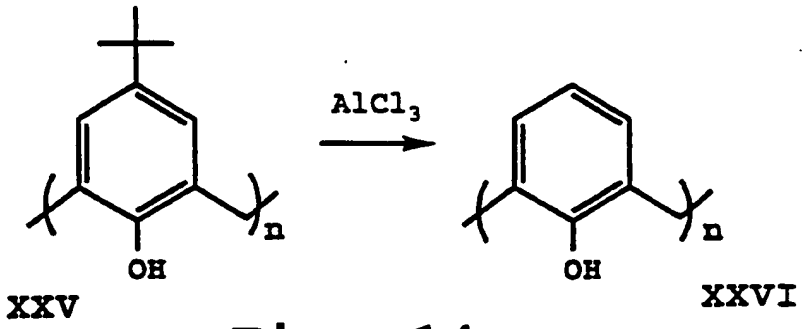


XVI

XXIII

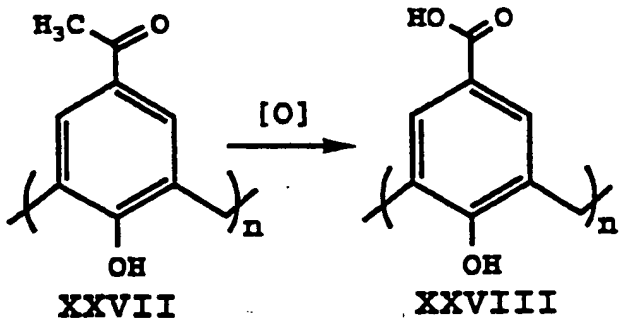
XXIV

Fig. 13



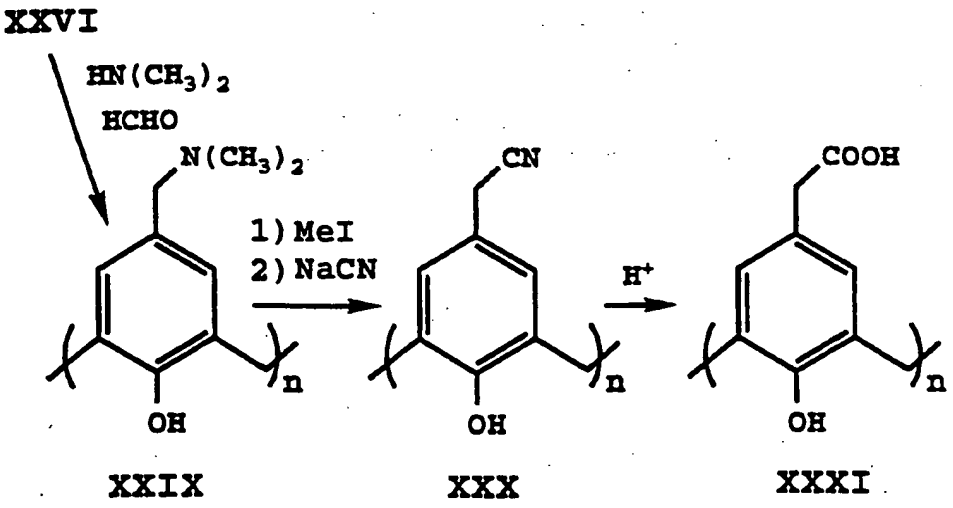
XXV XXVI

Fig. 14



XXVII XXVIII

Fig. 15



XXIX XXX XXXI

Fig. 16

Fig. 17

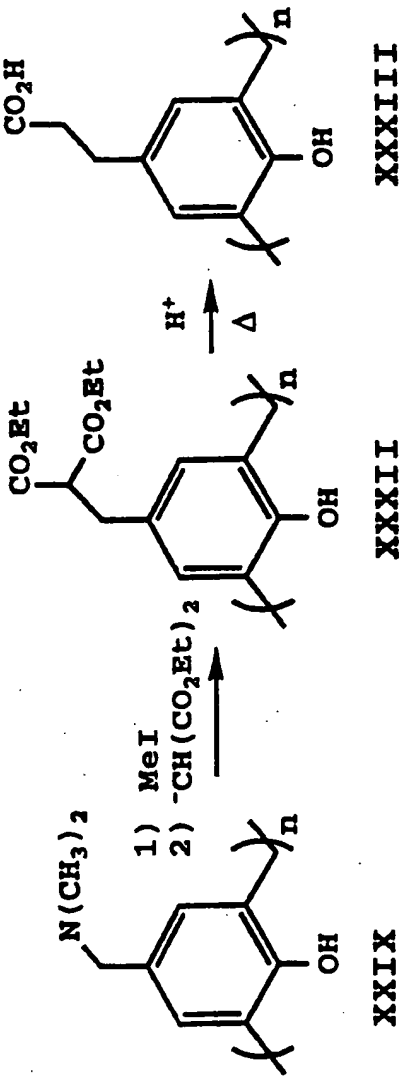
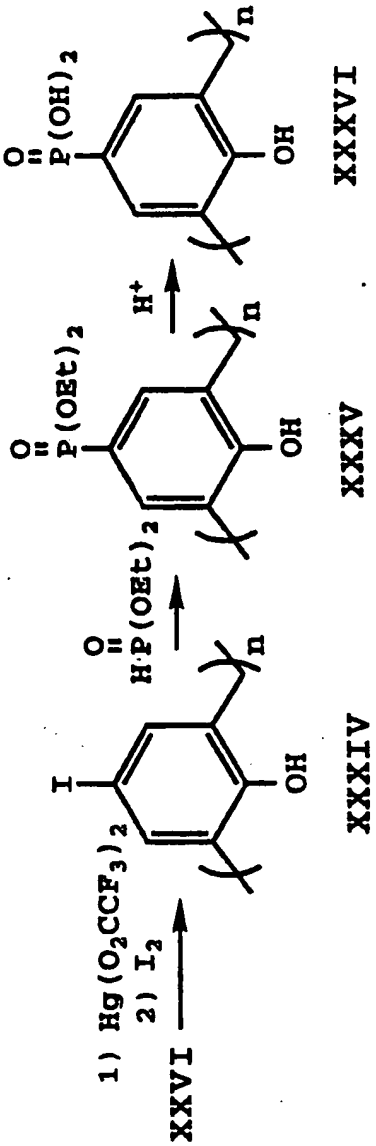

N(CH3)2
OH
n
1) MeI
2) -CH(CO2Et)2
CO2Et
CO2Et
H+
Δ
CO2H
XXIX
XXXII
XXXIII

Fig. 18


1) Hg(O2CCF3)2
2) I2
XXVI
I
OH
n
O
H·P(OEt)2
O
P(OEt)2
H+
O
P(OH)2
XXXIV
XXXV
XXXVI

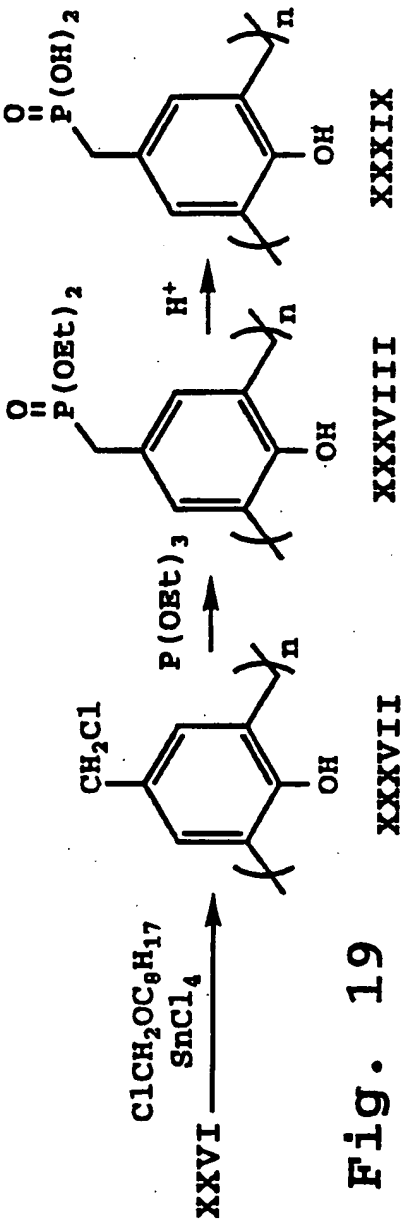

XXVI
ClCH2OC8H17
SnCl4
CH2Cl
OH
n
XXXVII
P(OEt)3
O
P(OEt)2
XXXVIII
H+
P(OH)2
XXXIX

Fig. 19

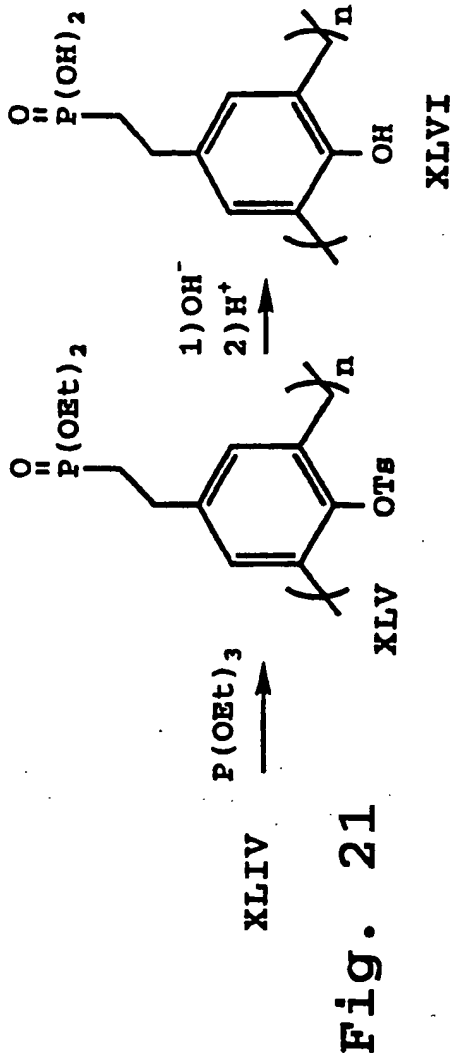

XLIV
P(OEt)3
OTs
XLV
1)OH-
2)H+
XLVI

Fig. 21

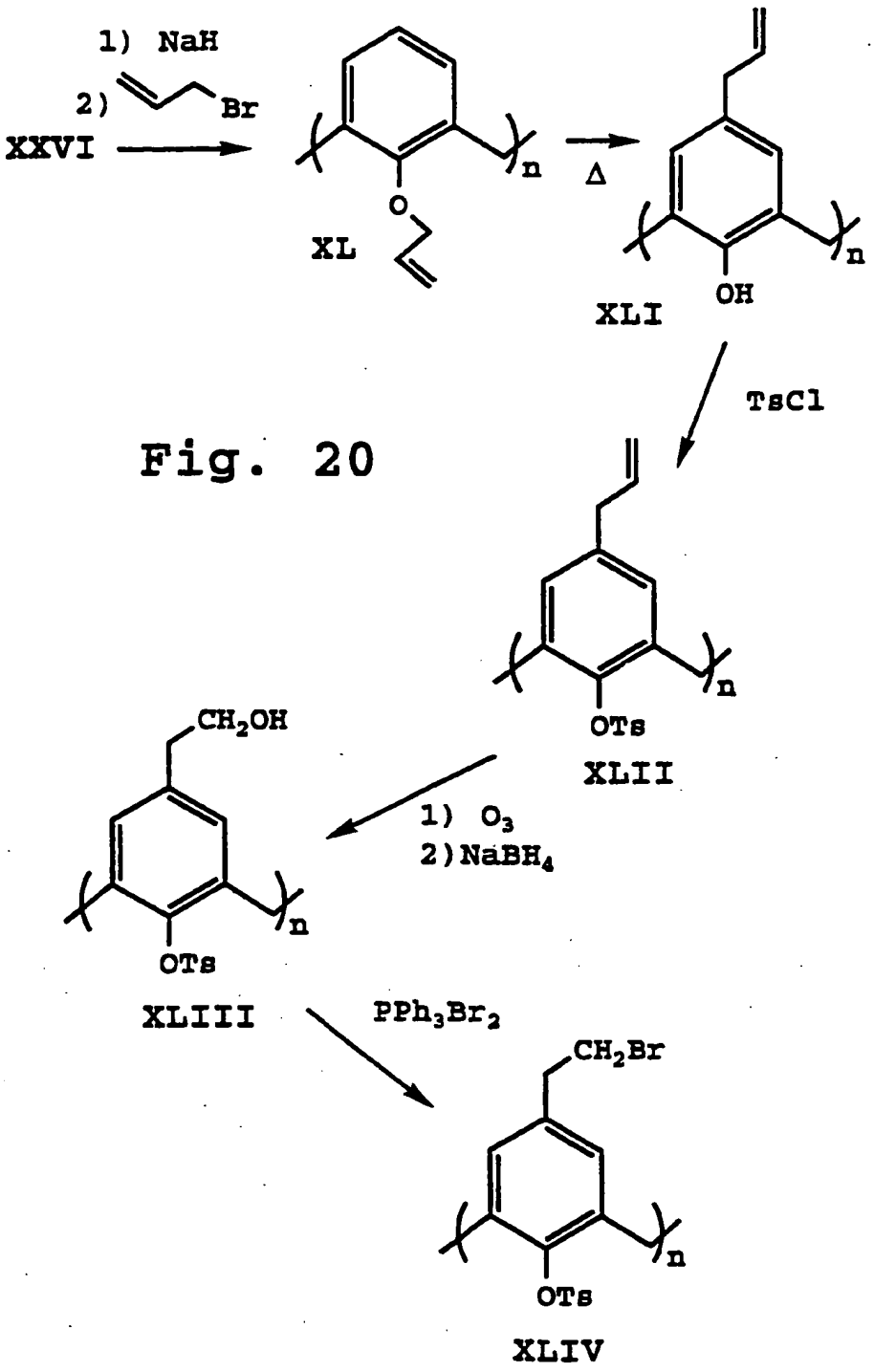
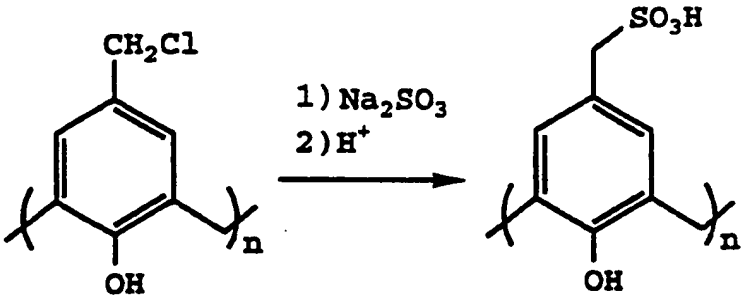
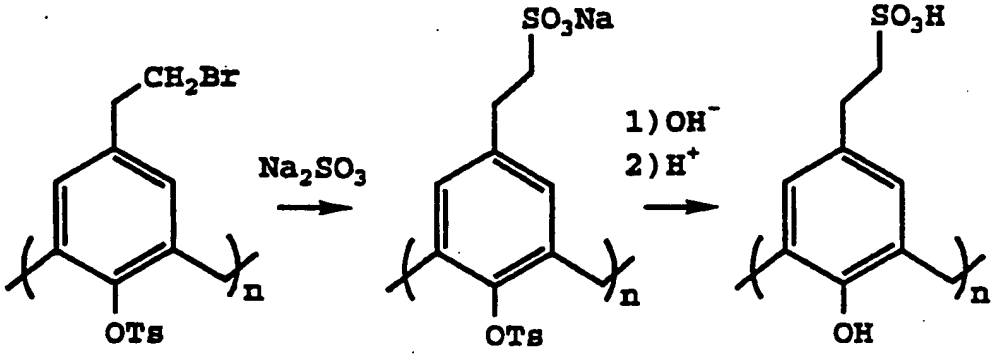

1) NaH
2)
Br
XXVI
n
O
XL
Δ
OH
XLI
TsCl
OTs
XLII
CH2OH
1) O3
2) NaBH4
XLIII
PPh3Br2
CH2Br
XLIV

Fig. 20



XXXVII XLVII

Fig. 22



XLIV XLVIII XLIX

Fig. 23

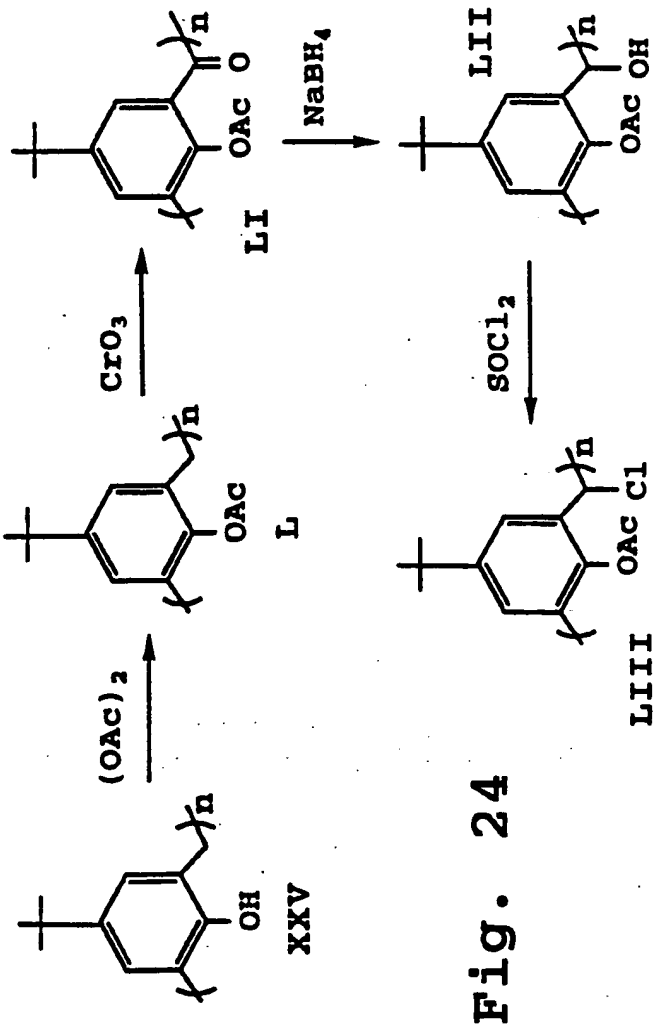

XXV
L
LI
LII
LIII
LV
LVI
(OAc)2
CrO3
NaBH4
SOCl2
AlCl3
1) CN-
2) H+
OH
OAc
OAc O
OAc OH
OAc Cl
OH CO2H
n

Fig. 24

Fig. 25

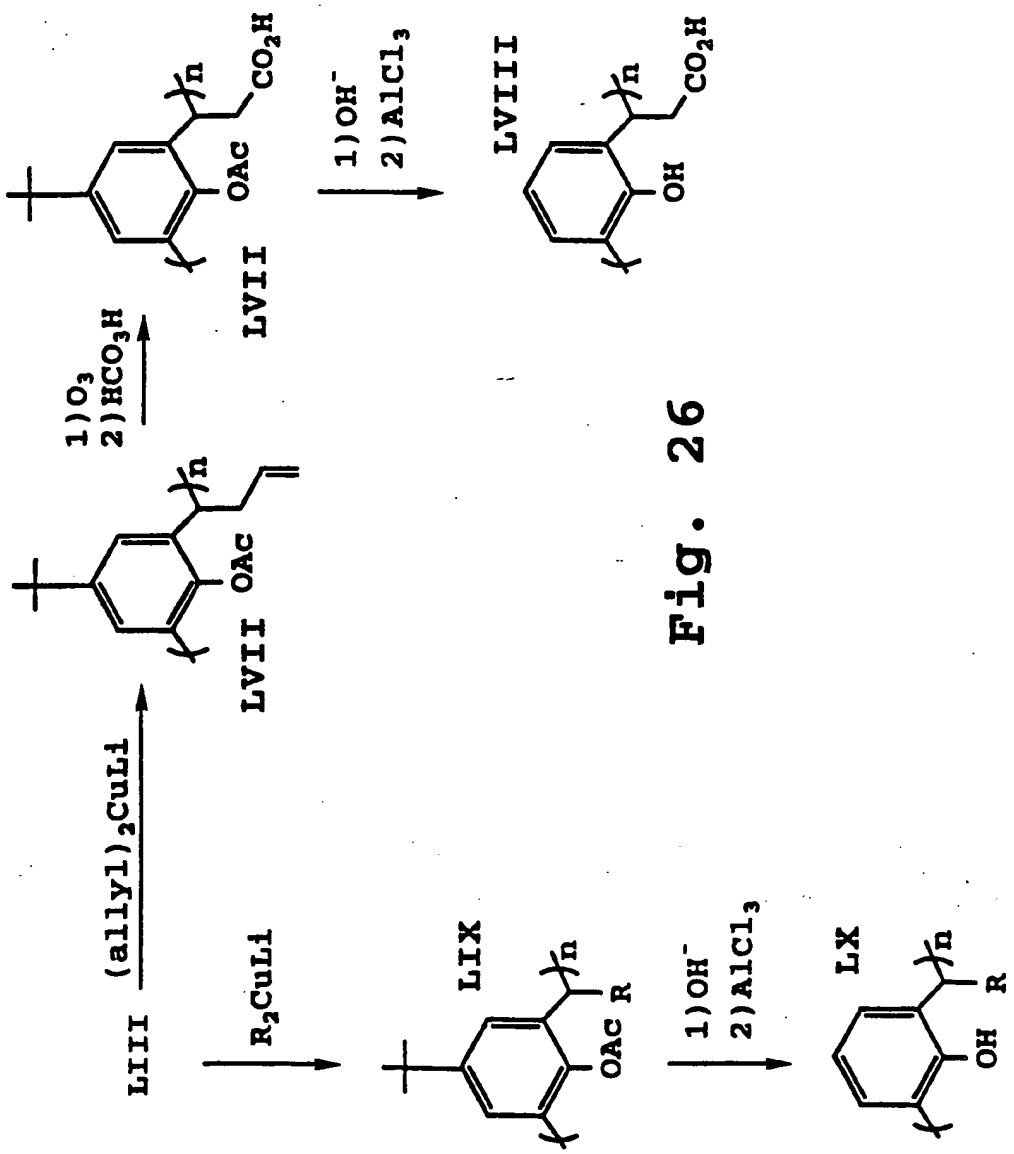

LIII
(allyl)2CuLi
R2CuLi
LIX
1)OH-
2)AlCl3
LX
LVII
1)O3
2)HCO3H
LVII
1)OH-
2)AlCl3
LVIII

Fig. 26

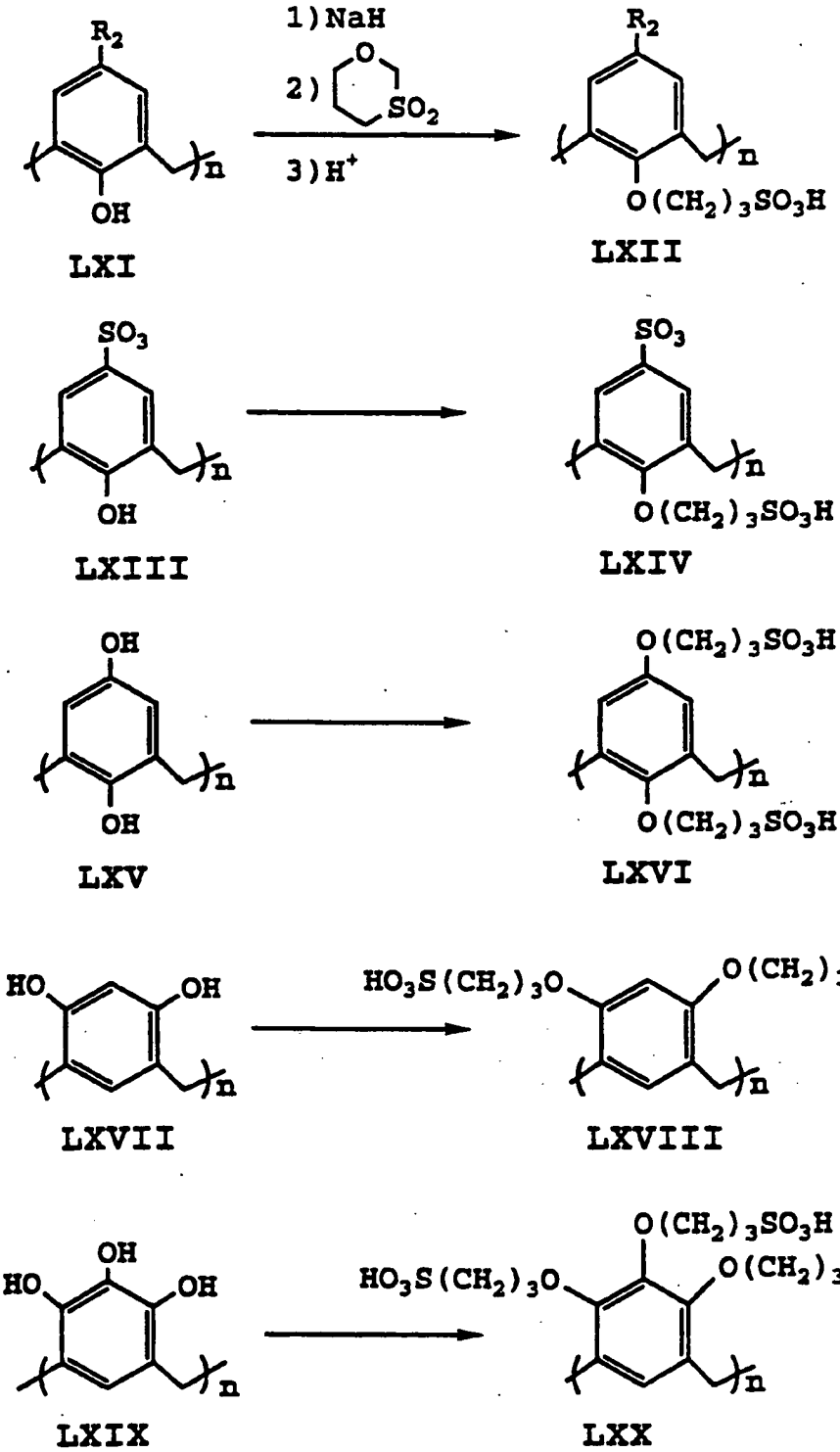


Fig. 27

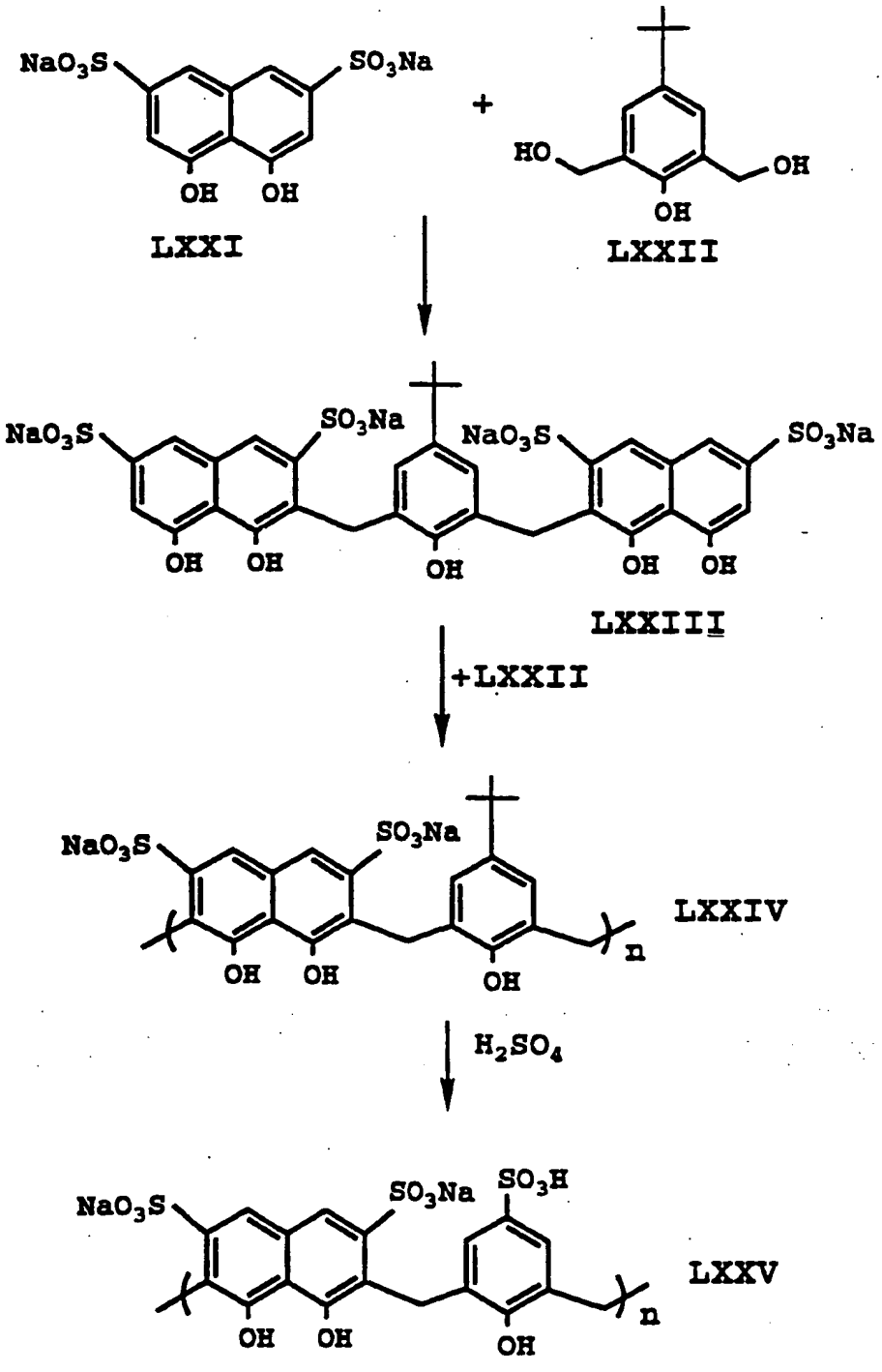

NaO3S
SO3Na
OH
OH
LXXI
+
HO
OH
OH
LXXII
NaO3S
SO3Na
NaO3S
SO3Na
OH
OH
OH
OH
OH
LXXIII
+LXXII
NaO3S
SO3Na
OH
OH
OH
n
LXXIV
H2SO4
NaO3S
SO3Na
SO3H
OH
OH
OH
n
LXXV

Fig. 28

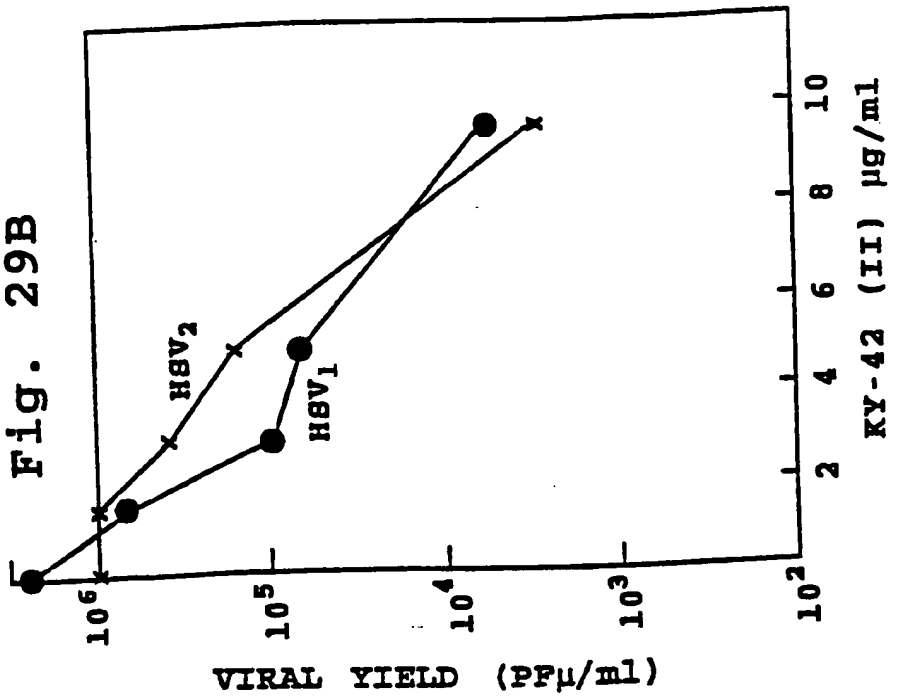


Fig. 29B
HSV2
HSV1
KY-42 (II) µg/ml
2 4 6 8 10
10^6 10^5 10^4 10^3 10^2
VIRAL YIELD (PFµ/ml)

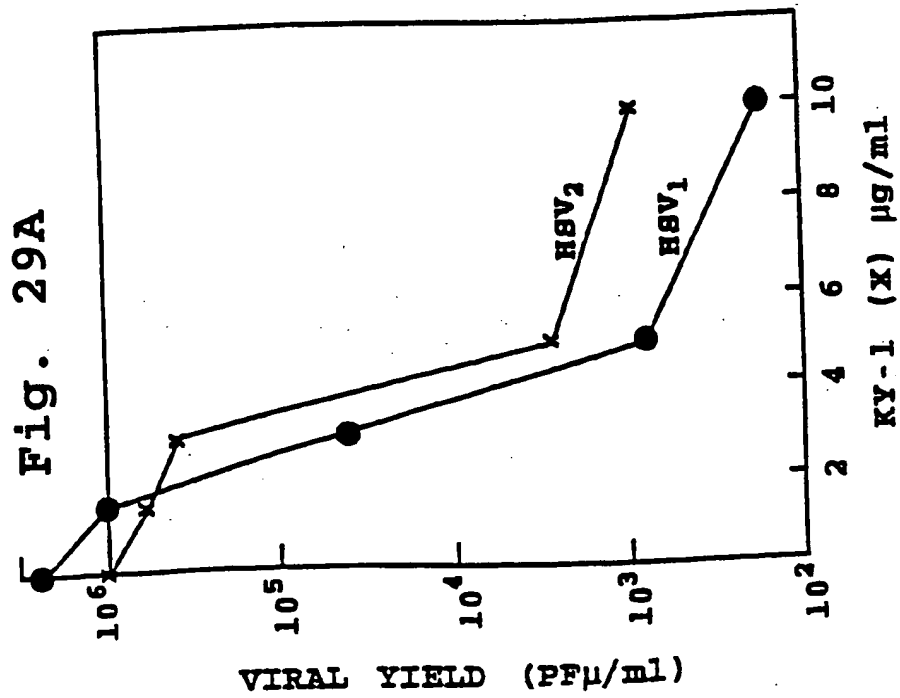


Fig. 29A
HSV2
HSV1
KY-1 (X) µg/ml
2 4 6 8 10
10^6 10^5 10^4 10^3 10^2
VIRAL YIELD (PFµ/ml)

Fig. 30

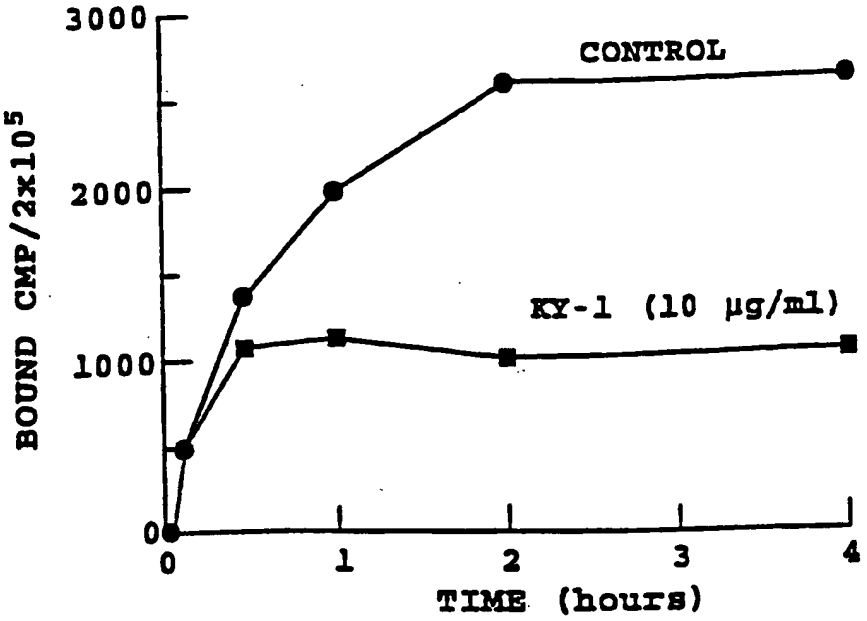

3000
2000
1000
0
BOUND CMP/2x10^5
CONTROL
KY-1 (10 µg/ml)
0
1
2
3
4
TIME (hours)

Fig. 31

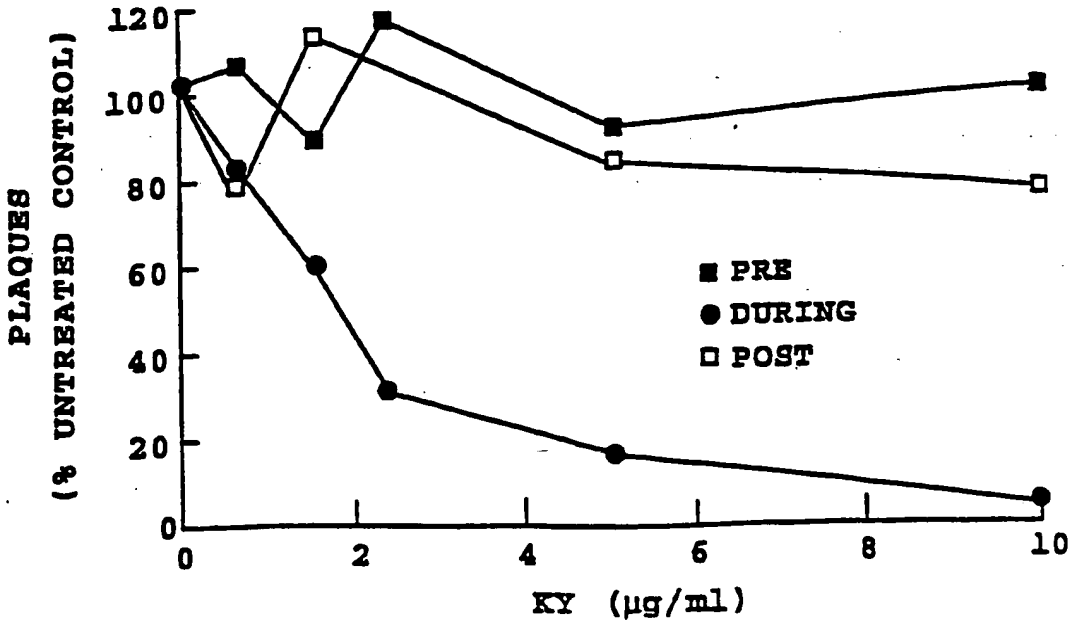

120
100
80
60
40
20
0
PLAQUES
(% UNTREATED CONTROL)
PRE
DURING
POST
0
2
4
6
8
10
KY (µg/ml)

Fig. 32A

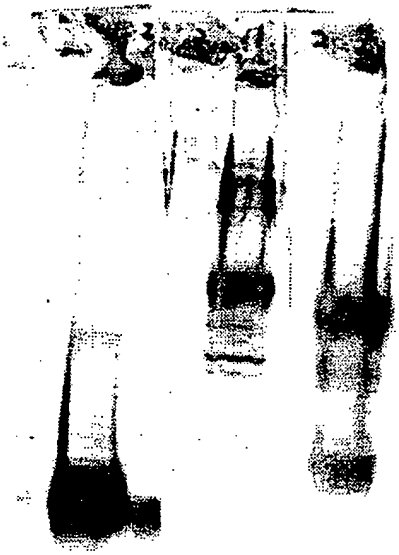


Fig. 32B

A B C D E F
D B C

Fig. 33A

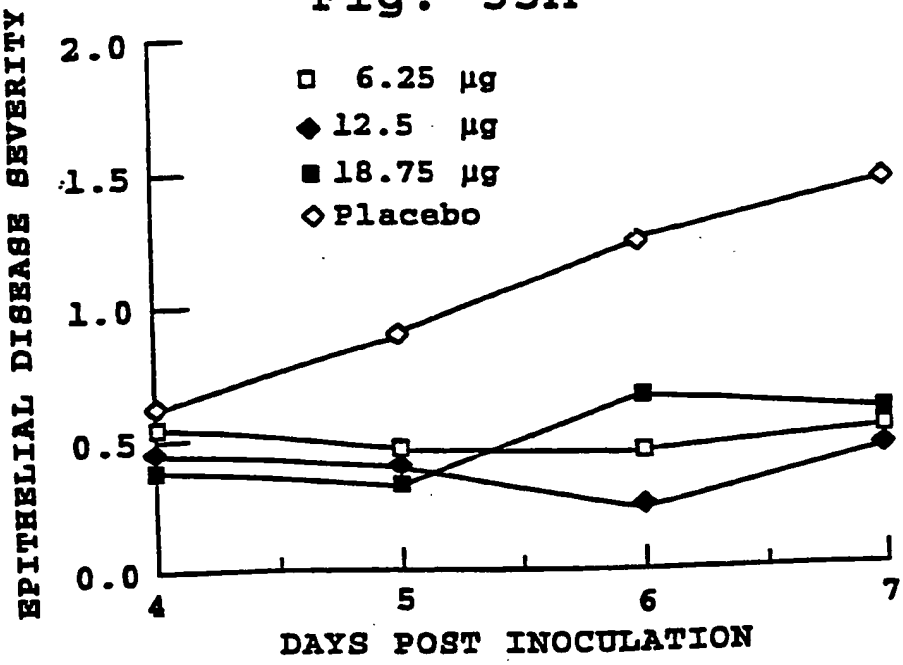


Fig. 33B

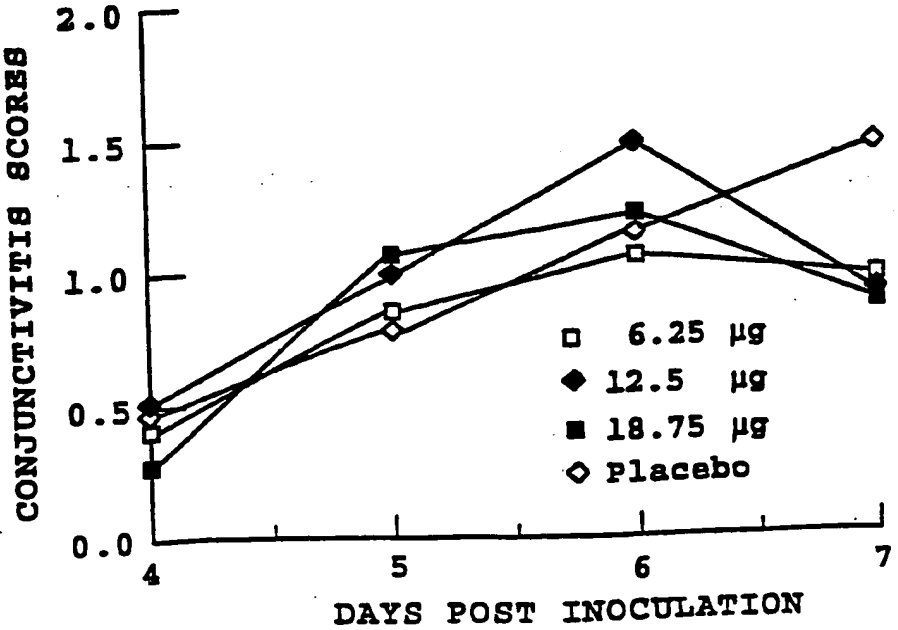


Fig. 33C

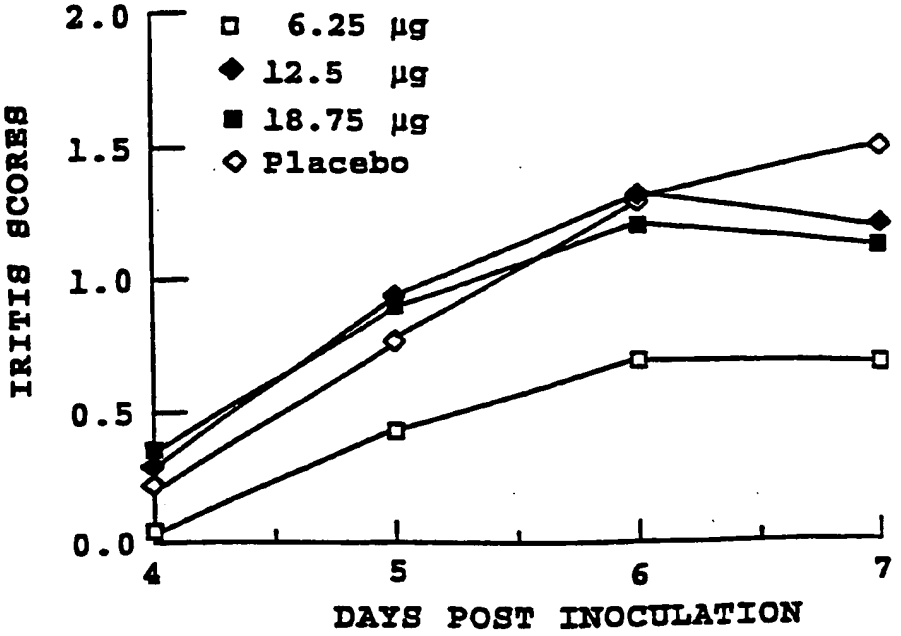
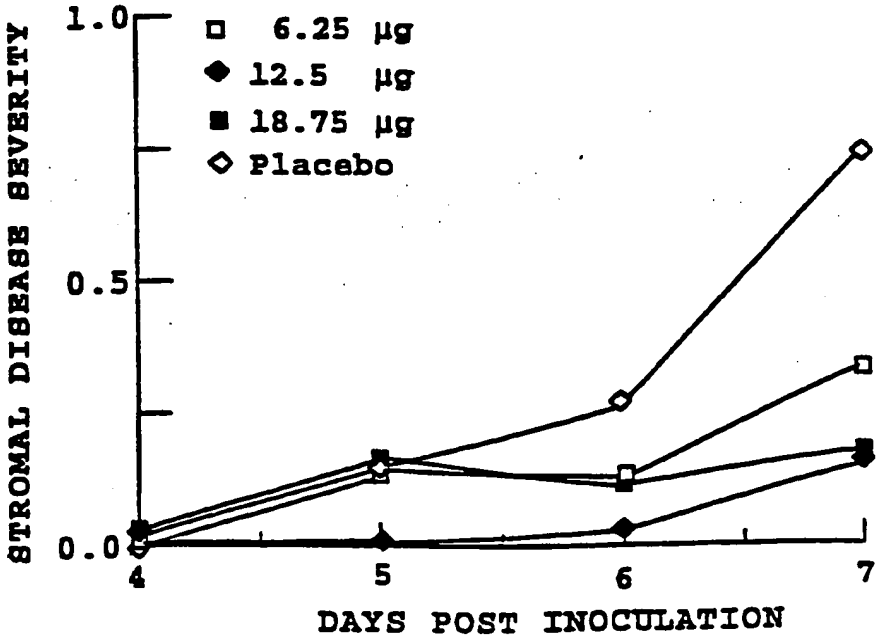


Fig. 33D



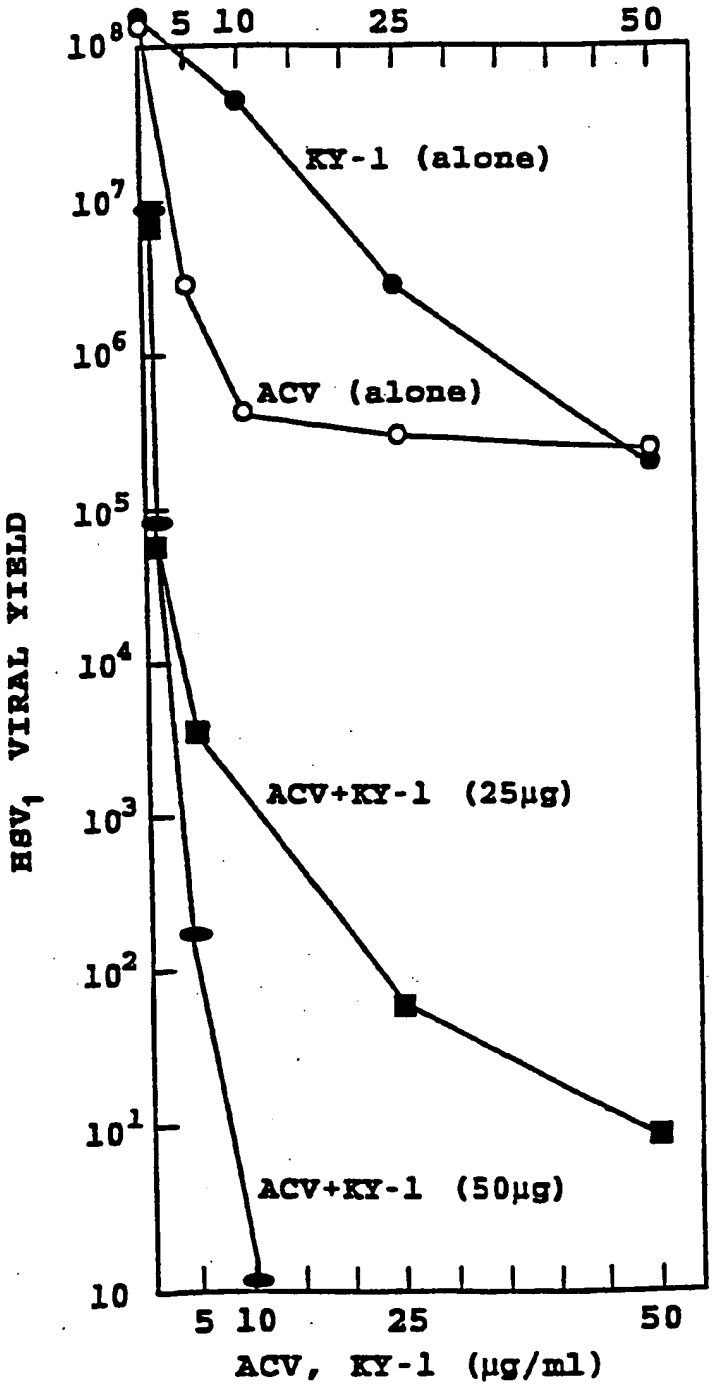

5 10 25 50
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10
KY-1 (alone)
ACV (alone)
ACV+KY-1 (25µg)
ACV+KY-1 (50µg)
HSV_1 VIRAL YIELD
5 10 25 50
ACV, KY-1 (µg/ml)

Fig. 34A

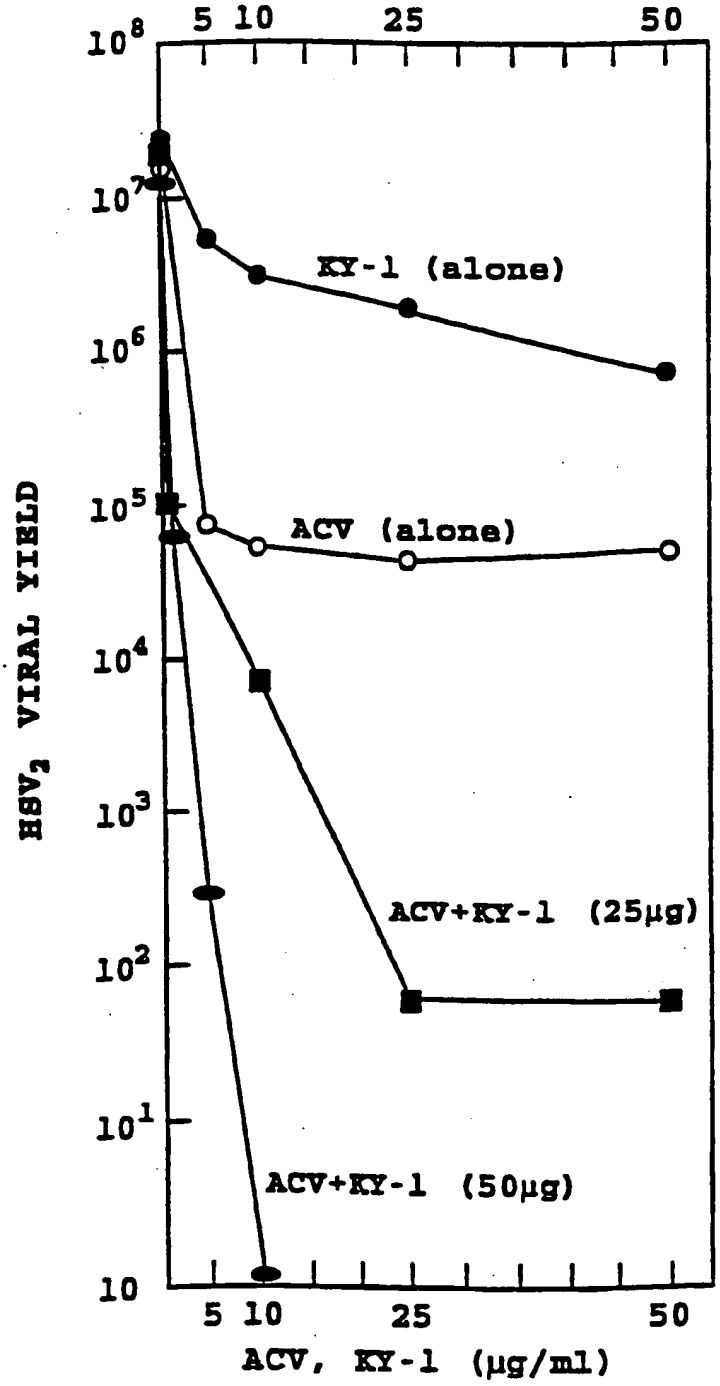

5 10 25 50
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10
KY-1 (alone)
ACV (alone)
ACV+KY-1 (25µg)
ACV+KY-1 (50µg)
HSV_2 VIRAL YIELD
5 10 25 50
ACV, KY-1 (µg/ml)

Fig. 34B

INTERNATIONAL SEARCH REPORT

[redacted] nal Application No
[redacted]3/07366

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 A61K31/18 A61K31/185 A61K31/255 A61K31/795 A61K31/80
A61K31/765 A61K31/19 A61K31/66 C07C309/24 C07C143/00
C07C311/13 C07F9/38 C07F9/40 C07C65/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 A61K C07C C07F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,92 12709 (GENELABS) 6 August 1992 cited in the application see abstract; claims	1-8, 13-26, 29-34
P,X	ANTIVIRAL RESEARCH vol. 20, no. S1 , 1993 page 105 W.CHOY ET AL. 'CALIX(n)ARENE SULFONATES AS NOVEL ANTIVIRAL AGENTS' & SIXTH INTERNATIONAL CONFERENCE ON ANTIVIRAL RESEARCH April 1993 , ITALY see the whole document	1-4,13, 14, 17-25, 30-34

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 November 1993	[illegible]

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	HOFF, P

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,90 00596 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 25 January 1990 see abstract; claims	1-4, 9-14, 17-24, 30-34
P,X	WO,A,92 13542 (RHONE-POULENC RORER) 20 August 1992 see abstract; claims	1-4, 9-14, 17-24, 30-34
Y	US,A,4 604 404 (MUNSON ET AL.) 5 August 1986 see the whole document	1-4,13, 14, 17-24, 30-34
Y	J. AM. CHEM. SOC. vol. 108 , 1986 pages 2409 - 2416 S. SHINKAI ET AL. 'HEXASULFONATED CALIX(6)ARENE DERIVATIVES: A NEW CLASS OF CATALYSTS, SURFACTANTS, AND HOST MOLECULES' see the whole document	1-4,13, 14, 17-24, 30-34 25
Y	J. CHEM. SOC. PERKIN TRANS. I 1987 pages 2297 - 2299 S. SHINKAI ET AL. 'NEW SYNTHESES OF CALIXARENE-p-SULPHONATES AND p-NITROCALIXARENES' cited in the application see the whole document	1-4,13, 14, 17-24, 30-34 25
Y	SYNTHESIS AND REACTIVITY IN INORGANIC AND METAL-ORGANIC CHEMISTRY vol. 21, no. 8 , 1991 pages 1231 - 1241 M. YILMAZ ET AL. 'SYNTHESIS OF NEW SUBSTITUTED CALIX(4)ARENES AND THEIR COMPLEXES WITH IRON(III)' cited in the application see the whole document	1-4,9, 10,13, 14, 17-24, 30-34 25,27,29
Y	PATENT ABSTRACTS OF JAPAN vol. 1, no. 4543 28 February 1989 & JP,A,02 229 198 (KANEBO LTD) 11 September 1990 see abstract	1-4, 11-14, 17-24, 30-34 25,28

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. ORG. CHEM. vol. 55 , 1990 pages 4487 - 4489 I. ALAM ET AL. 'CALIXARENES.24. COMPLEXATION BY WATER-SOLUBLE CALIXARENES' see the whole document	25,27,29
X	J. ORG. CHEM. vol. 42, no. 2 , 1977 pages 382 - 383 T.B. PATRICK ET AL. 'AN IMPROVED PREPARATION OF PHENOLIC(1.1.1.1.) METACYCLOPHANES' see the whole document	25,27,29
X	TETRAHEDRON vol. 45, no. 7 , 1989 pages 2177 - 2182 M. ALMI ET AL. 'CHLOROMETHYLATION OF CALIXARENES AND SYNTHESIS OF NEW WATER SOLUBLE MACROCYCLIC HOSTS' see the whole document	25,28
X	CHEMICAL ABSTRACTS, vol. 117, no. 16, 19 October 1992, Columbus, Ohio, US; abstract no. 160793y, KAWASA 'SPOT PREVENTION IN SILVER HALIDE PHOTOGRAPHIC MATERIAL' see abstract & JP,A,4 157 456 (KONICA CO.) 29 May 1992	25,26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 07366

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

REMARK: Although claims 1-24 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

[redacted] Application No
PCT/US 93/07366

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